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University
of Glasgow

**Understanding Recurrent
Vulvovaginal Candidiasis as a
dynamic biofilm disease of *Candida*
and *Lactobacillus***

Emily McKlound (BSc Hons)

Submitted in fulfilment of the requirements for the
Degree of Doctor of Philosophy

School of Medicine
College of Medical, Veterinary and Life Sciences
University of Glasgow

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Abstract

Despite strikingly high global prevalence of VVC, affecting up to 75% of women during their child-bearing years and resulting in up to 140 million episodes of RVVC, treatment options for women with azole-resistant isolates remain limited. Many women experience failed clinical treatment resulting in use of prolonged maintenance therapies for an excess of 6 months, which are often unsuccessful. Further, there is a clear lack of understanding in the literature pertaining to disease onset and potential mechanisms of treatment failure. Gaining a deeper knowledge of VVC is obstructed by controversy surrounding the presence of *Candida* biofilm on vaginal mucosa. As a result, there are currently no FDA-approved treatments for biofilm-related episodes. Additionally, a better understanding of the antagonistic interactions between *Candida* and vaginal *Lactobacillus* species could lead to the development of alternative probiotic therapies for the treatment of recurrent azole resistant VVC.

This PhD study aimed to assess a panel of clinical samples from healthy woman and those with RVVC to investigate the influence of *Candida*, the vaginal microbiome, and how microbial interactions influence disease pathology. A bibliometric analysis of literature pertaining to *Candida* and *Lactobacillus* biofilm research was carried out to assess caveats in current understanding. Clinical samples were assessed for *Candida* load and presence of biofilms using a range of microbiological techniques. *Candida* clinical isolates were screened for biofilm formation and antifungal susceptibility to frontline azole-treatments. 16S rRNA sequencing was used to characterise both the healthy and RVVC vaginal microbiome and to correlate patient metadata to microbial populations. Finally, RNA sequencing technology was utilised to identify potential mechanisms of antagonism between *C. albicans* and *L. crispatus* in an *in vitro* co-culture biofilm model.

The results of this study identified elevated *Candida* levels in RVVC and evidence of *Candida* biofilm formation in clinical samples of RVVC including presence of *C. albicans* hyphae and aggregation and detection of biofilm-related gene expression. Further, *Candida* clinical isolates were capable of heterogeneous biofilm formation and displayed increased antifungal tolerance. Analysis of microbial populations present in health and RVVC identified a partial replacement of health-associated *Lactobacillus* species including *L. crispatus* and *L. jensenii* by *L. iners* during RVVC.

Additionally, women with RVVC using hormonal contraceptives were found to have a more similar bacterial profile to healthy women, compared with women using other contraceptives. *C. albicans* biofilm reduction by *Lactobacillus* species was observed in an *in vitro* co-culture model. Further, RNA sequencing revealed an α -amino acid biosynthesis/breakdown pathway by which *L. crispatus* may out-compete *C. albicans* during VVC to re-establish vaginal health. Finally, bi-daily addition of *L. crispatus* was shown to reduce *C. albicans* composition within a complex biofilm model.

This study highlights the potential impact of *Candida* biofilm formation in RVVC and the importance of the consideration of biofilms for diagnosis and treatment. Additionally, it contributes to our current understanding of the vaginal microbiome during RVVC and antagonistic interactions between *Candida* and *Lactobacillus*. This work provides a foundation for future studies to further elucidate triggers for the development and recurrence of VVC/RVVC and the pathogenesis of the microbes involved. This and similar work will hopefully lead to the development of novel, more appropriate prevention and treatment options for persistent, azole resistant VVC.

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Author's declaration

I declare that I have carried out the work described in this thesis unless otherwise acknowledged or cited, under the supervision of Professor Gordon Ramage, Professor Marcello Riggio and Dr. Leighann Sherry. I further declare that this thesis has not been submitted for any other degree at the University of Glasgow, or any other institution.

A handwritten signature in blue ink, appearing to read 'Eme' followed by a large, stylized loop.

Emily McCloud

List of publications

Sherry, L., Kean, R., **McKloud, E.**, O'Donnell, L.E., Metcalfe, R., Jones, B.L. and Ramage, G., 2017. Biofilms formed by isolates from recurrent vulvovaginal candidiasis patients are heterogeneous and insensitive to fluconazole. *Antimicrobial agents and chemotherapy*, 61(9), pp.e01065-17.

McKloud E, Delaney C, Sherry L, Kean R, Williams S, Metcalfe R, Thomas R, Richardson R, Gerasimidis K, Nile CJ, Williams C, Ramage G. Recurrent Vulvovaginal Candidiasis: a Dynamic Interkingdom Biofilm Disease of *Candida* and *Lactobacillus*. *mSystems*. 2021 Aug 10:e0062221. doi: 10.1128/mSystems.00622-21.

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Appendix iii: Patient consent form

Appendix iv: Patient questionnaire

Appendix v: *C. albicans* SC5314 standard curve for qPCR calculations

Appendix vi: *E. coli* K12 standard curve for qPCR calculations

Appendix vii: Recurrent Vulvovaginal Candidiasis: A Dynamic Interkingdom Biofilm Disease of *Candida* and *Lactobacillus*

Abbreviations

ALS3: Agglutinin-like sequence 3
AMB: Amphotericin B
ASV: Amplicon sequence variants
BV: Bacterial vaginosis
CDR1: Cerebellar degeneration related protein 1
CFE: Colony forming equivalent
CFS: Cell-free supernatant
CFU: Colony forming unit
CFW: Calcofluor white
CMI: Cell-mediated immunity
CST: Community state types
CVL: Cervico-vaginal lavage
ECM: Extracellular matrix
ELISA: Enzyme-linked immunosorbent assay
GP: General practitioner
H₂O₂: Hydrogen peroxide
HRT: Hormone replacement therapy
HVS: High vaginal swab
HWP1: Hyphal wall protein 1
IUD: Intrauterine device
LAB: Lactic-acid bacteria
MALDI-TOF: Matrix Assisted Laser Desorption/Ionization – Time Of Flight
MDR1: Multidrug resistance 1
MIC: Minimum inhibitory concentration
MRS: De Man, Rogosa and Sharpe
NAC: Non-albicans species
NGS: Next generation sequencing
NTC: No-template controls
ONT: Oxford Nanopore Technologies
OTU: Operational taxonomic unit
PBS: Phosphate buffered saline
PCA: Principal components analysis
PCoA: Principal coordinates analysis
PCR: Polymerase chain reaction

PEA: Proximity extension assay

PMA: Propidium monoazide

PMNs: Polymorphonuclear neutrophils

ROS: Reactive oxygen species

RPML: Roswell Park Memorial Institute

RVVC: Recurrent vulvovaginal candidiasis

SAB: Sabouraud dextrose agar

SAP: Secreted aspartyl proteinase

STI: Sexually transmitted infection

VVC: Vulvovaginal candidiasis

WHO: World Health Organisation

XTT: [2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide]

YPD: Yeast peptone dextrose

1 Introduction

Fungal infections are becoming increasingly recognised as a substantial health burden on the global population. Over 1 billion people are estimated to suffer from fungal infections each year, resulting in over 1.5 million deaths (Bongomin et al., 2017). These infections are commonly associated with mucosal sites such as the vagina and oral cavity, and though not life threatening, are associated with high rates of morbidity. It is estimated that most women will suffer from at least one episode of vaginitis in their lifetime, making it the most common complaint from women attending General Practitioner (GP) clinics (Sobel, 1997). An estimated 75% of women will suffer from vulvovaginal candidiasis (VVC) in their childbearing years with up to 8% developing recurrent VVC (RVVC), defined as four or more cases within one year (Sobel, 1992, Sobel et al., 1998). These recurrent cases are debilitating, impact quality of life and can result in significant mental health implications. VVC has been linked to use of antibiotics and contraceptives as well as new sexual partners, however it is not considered a sexually transmitted disease and no distinguishable cause has been identified.

The biofilm-forming yeast *Candida albicans* is reported as the predominant pathogen responsible for up to 90% of VVC cases (Sobel et al., 1998). *Candida* species, predominantly *C. albicans*, form thick biofilms that dramatically increase fungal tolerance to drugs commonly used in the treatment of VVC such as fluconazole, flucytosine and amphotericin B (AMB) (Baillie and Douglas, 2000). Despite the identification of *Candida* biofilms on vaginal mucosa, the predicted therapeutic challenge this presents in VVC is still disputed (Swidsinski et al., 2019). VVC is a significant burden both economically and for women's health and its prevalence is poorly documented globally due to high levels of self-treatment. Identifying triggers for development and recurrence of VVC and the pathogenesis of the microbes involved could considerably improve prevention and treatment options for women with recurrent, azole-resistant cases.

This chapter reviews literature surrounding current understanding of VVC and the vaginal environment during health and disease as well as investigating current treatment options and highlighting the clinical importance of biofilm-induced antifungal-resistance in *Candida*.

1.1 Vaginitis

Vaginitis is a major cause of morbidity within the female population and is predominantly caused by bacterial vaginosis (BV) (40-45%), VVC (20-25%) and trichomoniasis (15-20%) (Paladine and Desai, 2018). An imbalance of the vaginal microbiome resulting in overgrowth of pathogenic microbes is thought to contribute to onset of vaginitis. BV is associated with a decrease or absence of protective lactobacilli species in the vagina and overgrowth of the biofilm-forming anaerobic coccobacillus, *Gardnerella vaginalis*. *G. vaginalis* is frequently asymptotically present in the vagina but can form dense biofilms, releasing lysogenic toxins which result in symptoms of disease (Patterson et al., 2007b). Treatment with metronidazole is recommended for BV, however, 20-40% of patients suffer from recurrent cases within one month of treatment (Danielsson et al., 2011). The reason for these recurrent episodes is often attributed to increased antimicrobial resistance attained as a result of bacterial biofilm formation. A common side-effect of BV antibiotic treatment is the onset of the fungal syndrome, VVC.

VVC, commonly referred to as vaginal thrush, is a result of overgrowth of *Candida* in the vagina. Current understanding of VVC onset involves dysbiosis of the vaginal microbiome as well as atypical host immune responses to *Candida*, however this is poorly understood.

1.1.1 Clinical significance

Although not associated with mortality, out of the 75% of women affected by VVC, 50% have at least one subsequent episode, with 5-8% developing RVVC (Sobel et al., 1998). The cause of recurrence of VVC is largely unknown and has been related to considerable morbidities including pelvic inflammatory disease, infertility and menstrual disorders. In addition to associated morbidities, the global market is also significantly impacted by VVC with an estimated annual cost of US \$600 million in 2013 for products such as Canesten (clotrimazole, Bayer, Leverkusen, Germany), fluconazole and itraconazole (Denning et al., 2018b). A novel drug, ibrexafungerp, has shown efficacy for the treatment of resistant episodes of RVVC which could greatly reduce the burden of VVC/RVVC in the future (Azie et al., 2020).

RVVC impacts patients' daily lives, with many women reporting loss of confidence, relationship difficulties and lower quality of life (Irving et al., 1998). One cross-sectional study of 44 women comparing the mental health of women with RVVC and those with no history of RVVC, reported significantly higher levels of clinical depression and poorer satisfaction with life in women with RVVC (Irving et al., 1998). Due to the nature of the disease, it is more likely that women would participate in online surveys as opposed to presenting at gynaecological clinics to partake in clinical studies. One such online survey was conducted to investigate the health of 620 women with RVVC in the USA, UK, France, Germany, Italy and Spain (Aballea et al., 2013). Results from this study found a 35% increase in the proportion of women with anxiety/depression in the RVVC cohort compared to the general population. Due to the nature of this study however, self-reported diagnoses could not be verified and therefore the findings could be exaggerated. Nonetheless, these studies highlight the multifactorial impact of RVVC on the global female population and the importance of effective management and treatment of RVVC.

While not fully characterised, onset of VVC is associated with overgrowth of *Candida* species in the vagina and risk factors include the use of antibiotics, pregnancy, immunosuppression, new sexual partners and hormone replacement therapy (HRT) (Denning et al., 2018b). Due to commensal carriage, isolation of *Candida* from the vagina in the absence of symptoms is not indicative of VVC. For this reason, diagnosis requires the presence of VVC-associated symptoms such as burning, itching, vulvar pruritis and cottage-cheese discharge. Symptoms can vary by case and as such, diagnosis relying on patient health, risk factors and clinical examination alone is problematic. Microscopy to identify yeast cells is often performed, however, positive identification of yeast cells or hyphae is only achieved in around 50% of cases (Mendling et al., 2012). Comparison of the accuracy of clinical diagnosis against DNA probing to diagnose VVC found sensitivity and specificity of detection to be 83.8% and 84.8%, respectively (Lowe et al., 2009). Diagnosis therefore requires the presence of clinical symptoms and positive microbiological culture or Gram stain of yeast cells and/or hyphae from vaginal or vulvar swabs (Gonçalves et al., 2016).

Failure of clinical treatment to effectively eradicate VVC, results in the development of RVVC, defined as four or more episodes of VVC within 12 months. The estimated incidence of RVVC is 138 million cases annually with many cases being considered chronic conditions requiring extended suppressive therapies to alleviate symptoms. Despite this high prevalence, the causes of failed treatment and subsequent recurrence remain largely speculative and includes factors such as use of antibiotics, levels of oestrogen and use of contraceptives (Pirodda and Garland, 2006, Nwokolo and Boag, 2000, Spinillo et al., 1993). Biofilms formed by *Candida* species have been suggested to be an underappreciated reservoir for failed clinical treatment in VVC due to their inherent resistance to azoles used in VVC treatment (Muzny and Schwebke, 2015, Sherry et al., 2017, Rodriguez-Cerdeira et al., 2019, Wu et al., 2019, Harriott et al., 2010b). However, some authors dispute the presence of *Candida* biofilms in the vagina despite biofilms being a key aspect of vaginitis in BV (Swidsinski et al., 2019, Sobel, 2015b, Hardy et al., 2017). Factors other than antifungal resistance have been suggested to be important including the role of polymicrobial interactions, tissue invasion, germ tube formation and immunological defects in the host (Talaie et al., 2017, Swidsinski et al., 2019). Nonetheless, the mechanisms by which *Candida* switches from asymptomatic commensal to pathogen remain undefined.

1.1.2 Epidemiology

VVC is not a reportable disease and is often self-treated using over-the-counter antifungal agents, so the exact prevalence and distribution of disease is impossible to determine. Additionally, many studies rely on self-diagnosis of VVC, symptoms of which can often be misdiagnosed with symptoms of BV. RVVC affects women worldwide and a recent study has estimated its global prevalence based on demographics of women in their childbearing years (Figure 1) (Denning et al., 2018a).

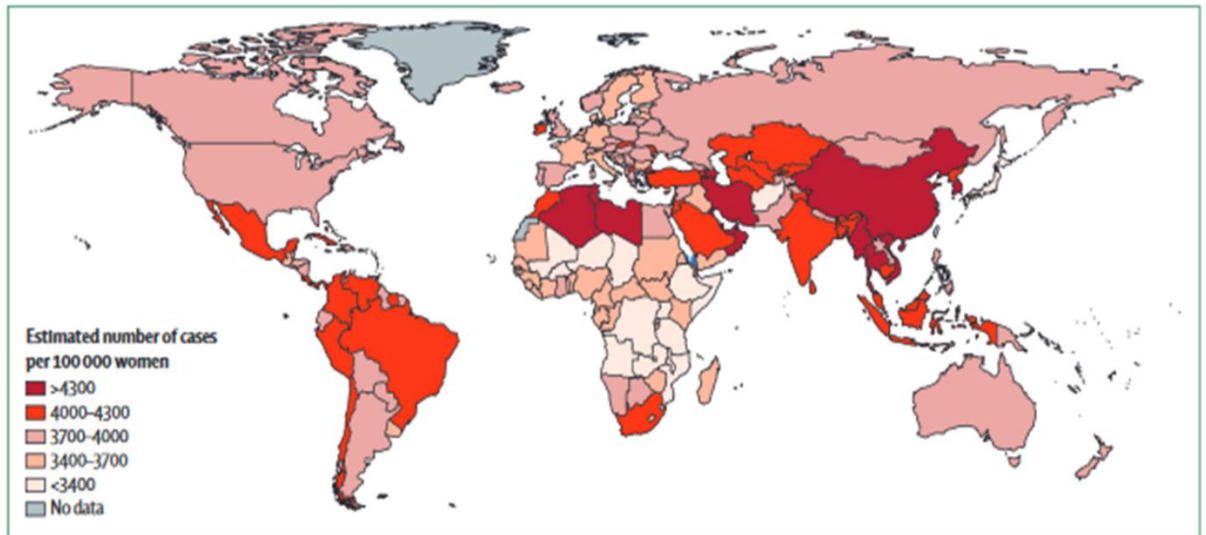


Figure 1.1 Global prevalence of RVVC per 100,000 women. Taken from (Denning et al., 2018b).

A study by Denning et. al. estimated the annual prevalence of RVVC to be >130 million cases, increasing to >150 million cases by 2030 (Denning et al., 2018a). The global incidence of RVVC is expected to increase due to the ageing female population and increase in the use of HRT in older women. This increase of RVVC cases is likely to be exacerbated in developing countries due to localised population growth and lack of accessible antifungal treatments. It is therefore critical that appropriate treatment and prevention methods are established to avoid increasing the burden of RVVC on healthcare facilities and the global market. Despite attempts to enumerate cases of VVC and RVVC, at present without sufficiently specific and sensitive diagnostic tools and with the availability of over-the-counter treatments, it remains impossible to determine an accurate prevalence for the disease worldwide.

Recent classification of the vaginal microbiome to unique community state types (CST) has provided a significant advance in vaginal research, allowing a standardised method of analysis across studies. The first article to identify these five classifications was a clinical study assessing the pH and microbiome of 394 pre-menopausal women from four ethnic groups (Asian, white, black and Hispanic) (Ravel et al., 2011a). CST are based on the most prevalent bacteria in each community and are defined as follows: I – *Lactobacillus crispatus*, II – *Lactobacillus gasseri*, III – *Lactobacillus iners* and V – *Lactobacillus jensenii*, summarised in Table 1.1. The fourth group is defined as a diverse group with no clear dominant species

and comprising various anaerobes including *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia* and *Mobiluncus*. Notably, this may not be indicative of higher diversity than other communities and may be attributed to the lack of a dominant species. These communities were used to determine interracial differences in vaginal microbiome; additionally, pH levels were observed among ethnic groups (Ravel et al., 2011a).

Table 1.1: pH of vaginal community groups in women of different ethnicities. Adapted from (Ravel et al., 2011a).

		Ethnic Groups				
Community groups		Asian	White	Black	Hispanic	All ethnic groups
I (<i>L. crispatus</i>)	Subjects	24	44	23	14	105
	pH	4.4 ± 0.52	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0
II (<i>L. gasseri</i>)	Subjects	5	8	5	7	25
	pH	4.4 ± 0.44	4.7 ± 0.44	5.0 ± 0.0	4.7 ± 0.22	5.0 ± 0.7
III (<i>L. iners</i>)	Subjects	41	26	33	35	135
	pH	4.0 ± 0.0	4.3 ± 0.30	4.0 ± 0.0	4.4 ± 0.59	4.4 ± 0.6
IV (Diversity Group)	Subjects	19	10	42	37	108
	pH	5.5 ± 0.44	5.5 ± 0.74	5.3 ± 0.44	5.3 ± 0.44	5.3 ± 0.6
V (<i>L. jensenii</i>)	Subjects	7	9	1	4	21
	pH	5.0 ± 0.89	4.85 ± 0.22	4.7 ± 0.44	5.0 ± 0.59	4.7 ± 0.4
All groups	Subjects	96	97	104	97	394
	pH	4.4 ± 0.59	4.2 ± 0.30	4.7 ± 1.04	5.0 ± 0.74	4.4 ± 0.7

The most common CST amongst the women involved in the study was CST III, which accounted for 34% of the total sample pool. The production of lactic acid was unanimous in all CST groups maintaining vaginal pH ~4.5, with a notably higher pH in CST IV, associated with lower levels of lactic-acid bacteria (LAB). This low vaginal pH is thought to be associated with maintenance of vaginal health through inhibition of non-LAB. Higher proportions of black and Hispanic women were characterised to the diverse CST (40.3% and 38.1%, respectively), suggesting a potential predisposition of these women to vaginal dysbiosis due to reduction in LAB. Conversely, it could be hypothesised that women of white and Asian decent may possess some level of protection against vaginitis due to vaginal colonisation with primarily *Lactobacillus* species.

1.1.3 Vaginal microbiome during health and VVC

The vaginal microbiome was first investigated by Döderlein in 1892 and was found to consist exclusively of Gram-positive bacilli, which are now known to be *Lactobacillus* species. Next-generation sequencing technologies have allowed greater depth of analysis into the microbial world to elucidate how immune tolerance and defence against potential pathogenic microorganisms is established. The vaginal microbiome is somewhat unique in that a low microbial diversity is associated with health. Vaginal dysbiosis, or onset on vaginitis, is therefore characterised by an increase in species diversity. It has become clear through microbiome analysis that there is no definitive health-associated vaginal microbiome. Instead, the five identified CSTs all represent a homeostatic environment with the functional capacity to prevent disease. In general, the vaginal microbiome of healthy premenopausal women is primarily comprised of lactobacilli species, namely *L. iners*, *L. crispatus*, *L. gasseri* and *L. jensenii* (Ravel et al., 2011b). Conversely, the microbiome during BV is well characterised showing a significant increase in microbial diversity compared with the low diversity found in healthy women (Liu et al., 2013b). This increase in diversity is coupled with a notable reduction in *Lactobacillus* and replacement with *G. vaginalis* resulting in biofilm formation, virulence and symptomatic BV (Vitali et al., 2015).

Studies have reported varied results when comparing the vaginal microbiome of women with VVC with healthy women. Some studies suggest that there is no evidence that the healthy microbiome protects against onset of disease (Zhou et al., 2009b). However, recent literature reports that disease is likely associated with a greater microbial diversity and loss of specific, protective lactobacilli species accompanied by predominance of BV-associated bacteria such as *G. vaginalis* and other anaerobes (Liu et al., 2013a, Jian et al., 2015). Where a clear shift towards dysbiosis of the microbiome is observed in BV and *Chlamydia trachomatis* infections, VVC research has identified subtle differences in bacterial diversity that suggests VVC as an intermediate state between health and disease (Ceccarani et al., 2019). Further, studies assessing bacterial abundances in VVC have not seen significant reductions in levels of *Lactobacillus* (Liu et al., 2013b, Sobel and Chaim, 1996). Instead, it is becoming increasingly recognised that the functional capacity of specific *Lactobacillus* species present in health is more important in protection

against VVC. A common factor in microbiome studies of VVC at species-level is the reduction of species including *L. crispatus*, *L. jensenii* and *L. gasseri* and increase in *L. iners* (Ceccarani et al., 2019, Fredricks et al., 2005, Wertz et al., 2008). This suggests a protective role of these specific species during health which is subsequently lost in disease.

1.1.4 Immunopathology of VVC

Our understanding of the role of the immune response in VVC has changed drastically over recent decades. Candidiasis at mucosal sites such as oropharyngeal and gastrointestinal infections are known to be more common in patients with impaired T-cell immune responses (Clift, 1984, Samaranayake, 1992). As such, early VVC research in the 1990's assumed that protection against vaginal candidiasis was due to T helper-1 (Th-1) and Th-2 cell-mediated immunity (CMI). However, various *in vitro* and animal studies investigating the role of CMI through T-cell infiltration have failed to identify any role for Th-1 or Th-2 cytokines in VVC (Taylor et al., 2000). Further, no evidence of increased *Candida*-specific antibodies in vaginal secretions has been found to suggest protection via humoral immunity (Wozniak et al., 2002). This resulted in the conclusion that, unlike *Candida* infections in other body sites such as the oral cavity, patients with compromised immune systems do not show increased susceptibility to VVC (Leigh et al., 2001).

More recently, an important study involving human participants that were intravaginally infected with live *Candida* observed a non-inflammatory immune response, with elevated levels of polymorphonuclear neutrophils (PMNs) in women with an increased fungal load (Fidel et al., 2004). As such it was hypothesised that VVC results from a lack of PMN-mediated killing of *Candida* cells in the vaginal mucosa. Further studies have strengthened this hypothesis showing activation of the *NLRP3* inflammasome causing release of inflammatory mediators such as alarmins and IL-1 β from vaginal epithelial cells, leading to PMN recruitment (Yano et al., 2012, Bruno et al., 2015). This results in further recruitment of these inflammatory mediators, ultimately leading to the hyperinflammation seen during VVC (Yano et al., 2018). A summary of the current proposed host immune response to VVC by (Rosati et al., 2020) can be observed in Figure 1.2.

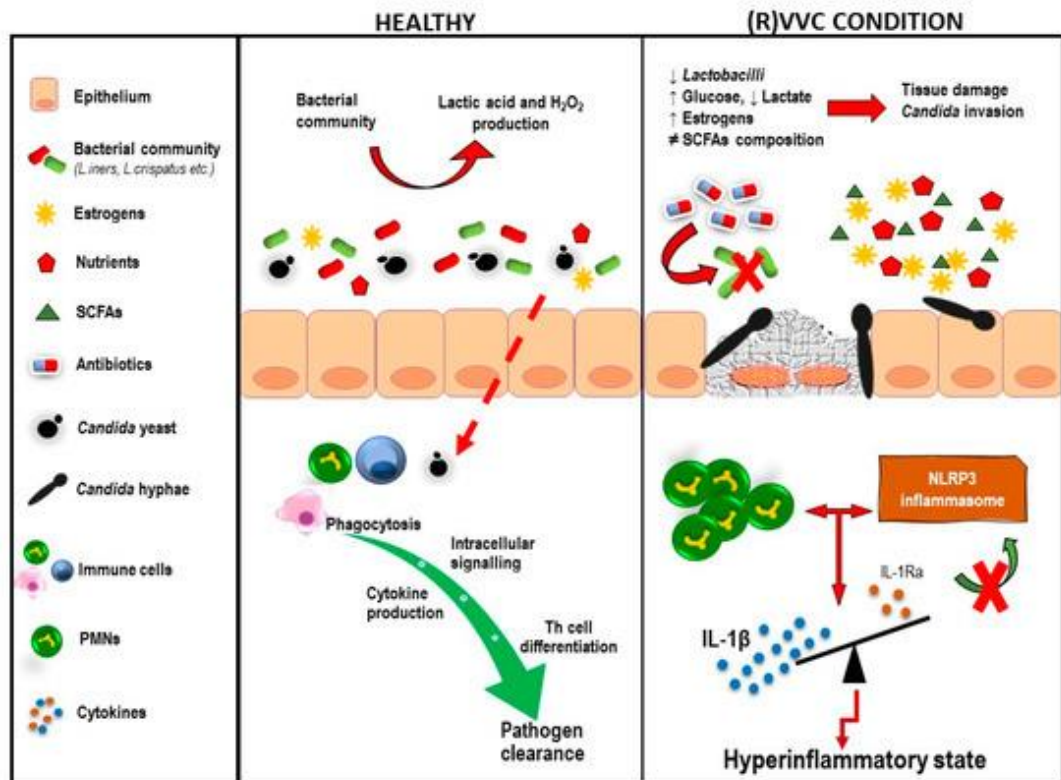


Figure 1.2: Immune response in RVVC. RVVC is a result of an exaggerated host immune response to environmental and immunological stimuli. Taken from (Rosati et al., 2020).

Therefore, it is currently hypothesised that VVC is not a result of an impaired adaptive immune response but is in fact due to an exaggerated innate response by PMNs. However, with evolving next-generation transcriptomic sequencing becoming increasingly available, it may be possible to further elucidate the immune mechanisms behind VVC pathogenesis.

1.2 *Lactobacillus*

Lactobacillus were first identified as the only bacteria present in the vaginal environment in 1892. Although it is now known that *Lactobacillus* species account for around 70% of the microbiome, their importance has been recognised throughout the 1900's and our understanding of these microbes continues to develop at present. The vaginal environment becomes colonised within 24 hours of birth and *Lactobacillus* become the predominant organism during puberty (Davis and Dobbing, 1974, Schwebke, 2001). This is due to increased glycogen levels of the vaginal epithelium as a result of increased oestrogen. *Lactobacilli* thrive in the

vaginal environment by metabolising glucose and maltose from glycogen breakdown via α -amylase and producing lactic acid, resulting in a protective acidic pH. Further, during menopause, a reduction in oestrogen levels reduces glycogen which lowers lactic acid production causing a rise in pH levels (Hickey et al., 2012). This ultimately reduces *Lactobacillus* levels in the vagina and has been shown to increase risk of infection (Burton and Reid, 2002). This highlights the importance of *Lactobacillus* species in vaginal health and their protective role throughout women's lives.

1.2.1 Role of *Lactobacillus* in vaginal health

In healthy women, LAB, predominantly lactobacilli, are thought to be responsible for maintaining a homeostatic microbiome by inhibiting growth and adhesion of other microbes via the production of secreted metabolites such as lactic acid, biosurfactants, bacteriocins and hydrogen peroxide (H_2O_2) (Morales and Hogan, 2010). However, the mechanisms behind the influence these metabolites have on *Candida* remains largely unknown.

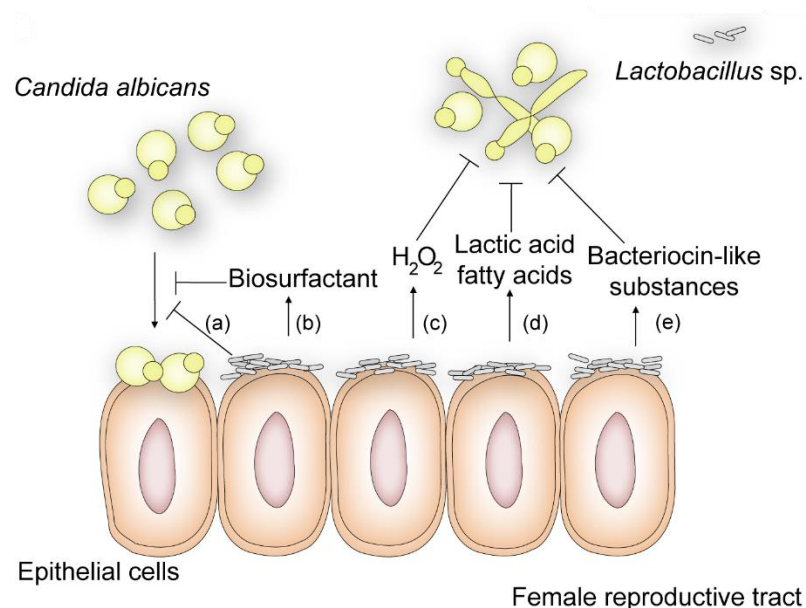


Figure 1.3: Commensal vaginal *Lactobacillus* inhibit adhesion of *Candida* to the vaginal epithelium via secreted metabolites. *Lactobacillus* inhibits *Candida* colonisation of vaginal mucosa through occupation of the niche and the production of biosurfactants. Additionally, *Lactobacillus* species lower the pH of the environment and produce H_2O_2 and bacteriocins which are inhibitory to *Candida*. Adapted from (Morales and Hogan, 2010).

The role of lactobacilli in vaginitis is uncertain; generally, they are thought to have a protective role and have been shown to prevent *C. albicans* adhesion to HeLa cells as well as enhancing recruitment of phagocytes and aiding in pro-inflammatory cytokine release (Matsuda et al., 2018b) (Rocha-Ramírez et al., 2017). A healthy vaginal flora, although not defined, is thought to consist mainly of H₂O₂-producing *Lactobacillus* species, maintaining a low, antimicrobial pH of <4.5. Infection is therefore a result of dysbiosis of this *Lactobacillus*-dominated environment, leading to loss of protective organisms and allowing overgrowth of disease-associated species. During BV, a notable reduction in *Lactobacillus* is observed with increased levels of *G. vaginalis*, *Mycoplasma hominis*, *Prevotella* spp., *Peptostreptococcus* spp., *Mobiluncus* spp., *Bacteroides* spp., *A. vaginae* and *Megasphaera* spp. (Cribby et al., 2008, Falagas et al., 2007). This observation is consistent and has resulted in an over-interpretation of the significance of reduced vaginal *Lactobacillus*. The Nugent criteria used to diagnose BV, assesses disease by the levels of *Lactobacillus* morphotypes present in vaginal fluid (Hickey et al., 2012). This observation does not account for the biological function of these species, and it may therefore be more appropriate to observe vaginal communities present to determine disease status.

Unlike BV, the dysbiotic event in VVC onset is largely unknown as studies have observed minimal reductions in *Lactobacillus* levels of women with VVC. Although it is hypothesised that VVC is due to a loss of *Lactobacillus*, the lowered pH of the *Lactobacillus*-dominated environment could result in *Candida* stress responses being triggered leading to hyphae formation and release of virulence factors causing dysbiosis of the microbiome. This was shown by Beyer and colleagues (Beyer et al., 2018b) where the MAP kinase CgHog1 of *Candida glabrata* was up-regulated in response to clinically relevant concentrations of lactic acid. CgHog1 increases the ability of *C. glabrata* to persist within the body, suggesting this could contribute to severity of VVC. However, the mechanistic trigger for VVC onset and failed treatment is likely multifactorial and remains undefined.

1.2.2 pH

The pH of the human body varies widely at different body sites from the highly acidic stomach pH of ~2, to the mildly acidic sites such as the skin and vagina, to neutral

and even alkaline in the saliva and colon, respectively. The pH of vaginal fluid can vary from around 6.6 to 4.2 depending on the presence of menstrual blood, however, it is typically sustained between 4 to 4.5 (Eschenbach et al., 2000). This acidic pH is thought to be maintained by the presence of LAB, namely *Lactobacillus* species. It is important to note that, although microorganisms are the primary source, vaginal epithelial cells also contribute to maintaining the acidic pH through the secretion of organic acids (Charlier et al., 2009, Boskey et al., 2001). During puberty, until menopause, increased levels of oestrogen results in high levels of glycogen deposits in the vagina (Paavonen, 1983). Glycogen is then broken down by α -amylase to glucose and maltose which is then anaerobically metabolised by commensals of the vaginal to produce acetic and lactic acid, lowering the pH and favouring LAB growth (Boskey et al., 1999). It is therefore during child-bearing years that the vagina is most acidic, and subsequently most inhibitory to pathogenic microbes.

The acidic pH of the vagina is associated with a decreased risk of chlamydia, trichomoniasis and has been shown to inhibit bacteria and inactivate viruses such as Herpes Simplex virus (Tuyama et al., 2006, Hanna et al., 1985). However, microorganisms such as *Candida*, found in many body sites, possess the ability to adapt to the external stimulus of environmental pH allowing for colonisation of the vagina. *C. albicans* can neutralise acidic and alkaline environments and has been shown to alter the pH by 3 units in 12 hours (Vylkova et al., 2011). This promotes fungal survival within the niche as well as allowing for hyphal morphogenesis and the production of virulence factors. Further, studies have reported an absence of lactic acid toxicity against *C. albicans* at acidic pH as low as 3 (Kasper et al., 2015a). *C. albicans* can also metabolise lactic acid in the presence of glucose (Lourenco et al., 2018). Therefore, evidence of *Candida* inhibition by *Lactobacillus* supernatants must be a result of other secreted metabolites present. However, although not directly inhibitory, concentrations of lactic acid present in the vagina (80mM) have been shown to increase susceptibility of *C. albicans* and *C. glabrata* to azole treatment (Lourenco et al., 2018).

1.2.3 D- and L- lactic acid

The presence of organic acids such as acetic and lactic acid, produced by LAB and vaginal epithelium when oestrogen is present, are hallmarks of vaginal health and are important for inhibition of pathogens and prevention of infection. *Lactobacillus* species are the main source of both D- and L-lactic acid production with the vaginal epithelium responsible for <15% of L-lactic acid production (Boskey et al., 2001). The D-lactic acid isomer provides greater protection against vaginal infection than the L-isomer (Witkin et al., 2013). *L. iners* is only capable of L-lactic acid production, unlike the other three commonly isolated *Lactobacillus* species outlined previously, which produce both isomers. The protective D-lactic acid isomer is most abundant in communities with high concentrations of *L. crispatus* and lowest when there are high levels of *L. iners*, *G. vaginalis* or *Streptococcus* (Witkin et al., 2013). The bactericidal properties of D-lactic acid have been widely studied showing it to enhance the protective properties of H₂O₂ and bacteriocins as well as inhibiting vaginal pathogens such as *G. vaginalis*, *Neisseria gonorrhoea* and *Trichomonas vaginalis* (Cadieux et al., 2009, Breshears et al., 2015). Further, lactic acid creates an anti-inflammatory environment by stimulating production of the anti-inflammatory cytokine IL-1RA in vaginal epithelium as well as suppressing the production of pro-inflammatory cytokines including IL-6 and TNF α (Hearps et al., 2017).

1.2.4 Hydrogen peroxide

H₂O₂ is another microbicidal secreted metabolite produced by many vaginal *Lactobacillus* spp. which is thought to contribute to the maintenance of vaginal health. H₂O₂ is an oxidising agent, toxic to catalase-negative bacteria, including anaerobes such as *G. vaginalis*, *N. gonorrhoeae* and *C. albicans* (Klebanoff et al., 1991, St Amant et al., 2002, Fitzsimmons and Berry, 1994). Studies have shown H₂O₂ production in the range of 1.01–15.50 μ g/mL in up to 95% of vaginal *Lactobacillus* strains capable of H₂O₂ production (*L. crispatus*, *L. acidophilus*, *L. fermentum* and *L. jensenii*) (Aslim and Kilic, 2006, Aroutcheva et al., 2001). Given its high production levels in the vagina and potent toxicity to vaginal pathogens, the use of H₂O₂ as a potential treatment for BV has been investigated. Two studies found vaginal irrigation with 3% H₂O₂ to sufficiently cure BV and aid in restoration of the healthy vaginal microbiome, proving efficacy of this potential treatment (Winceslaus and Calver, 1996, Cardone et al., 2003). Given that microbiome studies

of BV consistently find significant reductions in levels of H₂O₂-producing *Lactobacillus* species during disease (Ceccarani et al., 2019, Vitali et al., 2015), the effect of replacing the H₂O₂ produced by these key species appears to treat infection. One study investigating the effect of H₂O₂ concluded that it may protect against BV, but not VVC (Mijac et al., 2006). This conclusion was based on similar levels of H₂O₂ production in health and VVC. With development of sophisticated 16S rRNA and transcriptomic sequencing, it may now be more appropriate to consider the function capacity of the *Lactobacillus* species present during health and VVC. Further, potential interactions between *Candida* and *Lactobacillus* species which may affect H₂O₂ production should be considered.

The antimicrobial properties of H₂O₂ in the vaginal environment have been questioned due to evidence of inactivation of H₂O₂ by vaginal secretions and semen. The addition of either cervicovaginal fluid or semen to H₂O₂ *in vitro* were found to inactivate antimicrobial properties of H₂O₂ against Herpes Simplex Virus, *N. gonorrhoeae*, or any of six BV-associated bacteria (O'Hanlon et al., 2010). Further, a condemning review of the potential antimicrobial properties of H₂O₂ was published concluding that it is implausible that H₂O₂ has any antimicrobial effect in the vaginal environment (Tachedjian et al., 2018). However, it is important to note that none of these studies have considered the potential antifungal effect of H₂O₂ during VVC and, for this reason, it is still of clinical importance to consider the presence of H₂O₂-producing *Lactobacillus* species in VVC.

1.2.5 Bacteriocins

Bacteriocins are antimicrobial peptides produced by almost all LAB that provide these organisms with an ecological advantage for colonising body niches. Bacteriocins' mode of action involves pore formation and permeabilization of the cell membrane of competing organisms leading to amino acid and ion efflux, resulting in cell death (Gillor et al., 2005). These secreted metabolites have been extensively studied in the development of *Lactobacillus* probiotics. One bacteriocin, Nisin, produced by *Lactococcus lactis subsp. lactis* has been FDA-approved for use as a food preservative (Cleveland et al., 2001). Although suggested as a potential contraceptive due to its spermicidal activity, it cannot be considered for vaginal treatment as it is strongly bactericidal to healthy vaginal *Lactobacillus* (Dover et al.,

2008, Aranha et al., 2004). Several bacteriocins from vaginally isolated *Lactobacillus* have been shown to possess antimicrobial activity against vaginal pathogens including *N. gonorrhoeae*, *G. vaginalis* and *C. albicans* (Table 1.2).

Table 1.2: Bacteriocins produced by vaginally isolated bacteria.

Taken from (Dover et al., 2008).

SPECIES	BACTERIOCIN	ACTIVITY AGAINST	REFERENCE
<i>Lactobacillus rhamnosus</i> 160	Lactocin 160	<i>Gardnerella vaginalis</i> <i>Prevotella bivia</i> <i>Peptostreptococcus anaerobius</i> <i>Peptostreptococcus assacharoliticus</i> <i>Micrococcus luteus</i> <i>Listeria monocytogenes</i>	Li et al., 2005 and Aroutcheva et al., 2001c
<i>Lactococcus lactis subsp. lactis</i> HV219	Bacteriocin HV219	<i>Enterococcus faecalis</i> <i>Escherichia coli</i> <i>Lactobacillus casei</i> <i>Listeria innocua</i> <i>Proteus vulgaris</i> <i>Pseudomonas aeruginosa</i>	Todorov et al., 2006
<i>Lactobacillus salivarius</i> CRL1328		<i>Enterococcus faecalis</i> <i>Enterococcus faecium</i> <i>Neisseria gonorrhoeae</i>	Ocana et al., 1999
<i>Lactobacillus pentosus</i> TV35b	Pentocin TV35b	<i>Clostridium sporogenes</i> <i>Clostridium tyrobutyricum</i> <i>Lactobacillus curvatus</i> <i>Lactobacillus fermentum</i> <i>Lactobacillus sake</i> <i>Listeria innocua</i> <i>Propionibacterium acidipropionici</i> <i>Candida albicans</i>	Okkers et al., 1999

Many investigations into the importance of bacteriocins in the vaginal environment focus on the impact they may have in BV (Kelly et al., 2003, DeZwaan et al., 2007). Therefore, natural growth inhibition of Gram-positive vaginal commensals, including *Streptococcus* and *Lactobacillus*, in response to vaginally produced bacteriocins,

has been hypothesised as a potential cause of vaginal dysbiosis leading to BV onset. It is important to note, however, that this interaction is also relevant to VVC as reduced *Lactobacillus* levels allow for *Candida* overgrowth and symptomatic VVC.

1.3 *Candida*

As discussed in previous sections, *C. albicans* is the most prevalent causative organism identified in VVC (Sobel et al., 1998). *C. albicans* is also commonly isolated from the vagina as an asymptomatic commensal with a carriage rate of up to 33% in women of reproductive age (Achkar and Fries, 2010b). *C. albicans* is an opportunistic pathogen capable of causing systemic bloodstream mycoses in immunocompromised patients with associated mortality rates of up to 70% (Moran et al., 2009). Other non-*albicans* *Candida* (NAC) species account for 10-20% of VVC cases and are associated with complicated VVC with less severe symptoms but higher recurrence rates (Fidel et al., 1999). The most common NAC isolated in European studies is *C. glabrata* (14%) followed by other species such as *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* (Corsello et al., 2003b). Recent studies have shown a change in epidemiology with *C. glabrata* and other NAC species being isolated in greater than 50% of cases and more in RVVC (Ahmad et al., 2009). This increase in prevalence of innately fluconazole-resistant *C. krusei* is particularly concerning for future treatment options.

Candida is capable of survival in many different environment and host niches including the oral cavity, gastrointestinal tract, and vagina. It can exist as both a commensal and pathogenic yeast through its ability to undergo morphological changes and form hyphae. This yeast form allows *Candida* to colonise the vagina asymptotically, without detection by the immune response. However, environmental stressors such as pH and bacterial competition cause a dimorphic switch in *Candida* to form hyphae (Noble et al., 2017). Hyphal morphogenesis allows *Candida* to form thick biofilms adhered to vaginal epithelium which penetrate vaginal tissues of women with VVC, causing associated local inflammation (Ramage et al., 2001b, Harriott et al., 2010b). *Candida* biofilm formation in the vagina, although disputed, has been shown previously along with deep hyphal penetration of vaginal

epithelium in both *in vivo* and *ex vivo* (Figure 1.4) (Wu et al., 2020, Harriott et al., 2010b, Cassone, 2015).

1.3.1 Biofilms

The mechanism by which VVC occurs in the host is undefined and controversial. There is suggestion the microbiome plays a role whereby *Candida* overgrows, competing with commensals and transitioning from saprophytic yeast cells to pathogenic biofilms. The National Institutes of Health has estimated that biofilms are responsible for up to 80% of all human infections, highlighting the importance of understanding and preventing biofilm formation. *Candida* species, predominantly *C. albicans*, form thick biofilms which dramatically increase fungal tolerance to drugs commonly used in the treatment of VVC such as fluconazole, flucytosine and AMB (Taff et al., 2013). Sessile cells have been shown to tolerate antifungal concentrations 1000-fold greater than their planktonic counterparts (Ramage et al., 2012b).

Of biofilm-forming *Candida* species, the formation of *C. albicans* biofilms are most well studied and have been shown to form in four distinct stages: adhesion, proliferation (or initiation), maturation, and dispersion (Figure 1.4). The first stage, adhesion, involves round yeast cells adhering to a biotic or abiotic surface. This adhesion is mediated through recognition of ligands on the surface such as fibrinogen and fibronectin, quorum sensing, and expression of EAP1 and the ALS-family of genes (ALS1-ALS4) (Parsek and Greenberg, 2005, Dranginis et al., 2007). Deletion of ALS1, ALS2 or ALS3 has been shown to eliminate *C. albicans* ability to adhere to surfaces, highlighting the importance of these genes in early biofilm development (Zhao et al., 2005, Oh et al., 2005). After formation of this basal layer of yeast cells during the adhesion stage, *C. albicans* cells begin to proliferate, remaining attached to the surface. In this stage, pseudohyphae and hyphae begin to form from these budding yeast cells and continue to form and elongate throughout biofilm development. *C. albicans* biofilms are generally considered to be mature after 24 hours of development and are characterised by a thick, complex 3-dimensional structure comprised of yeast cells and hyphal cells encapsulated in extracellular matrix (ECM) composed of carbohydrates, glycoproteins, lipids, and nucleic acids (Zarnowski et al., 2014). The final stage of dispersion results in the

observation and positive yeast culture from vaginal discharge of women with active VVC.

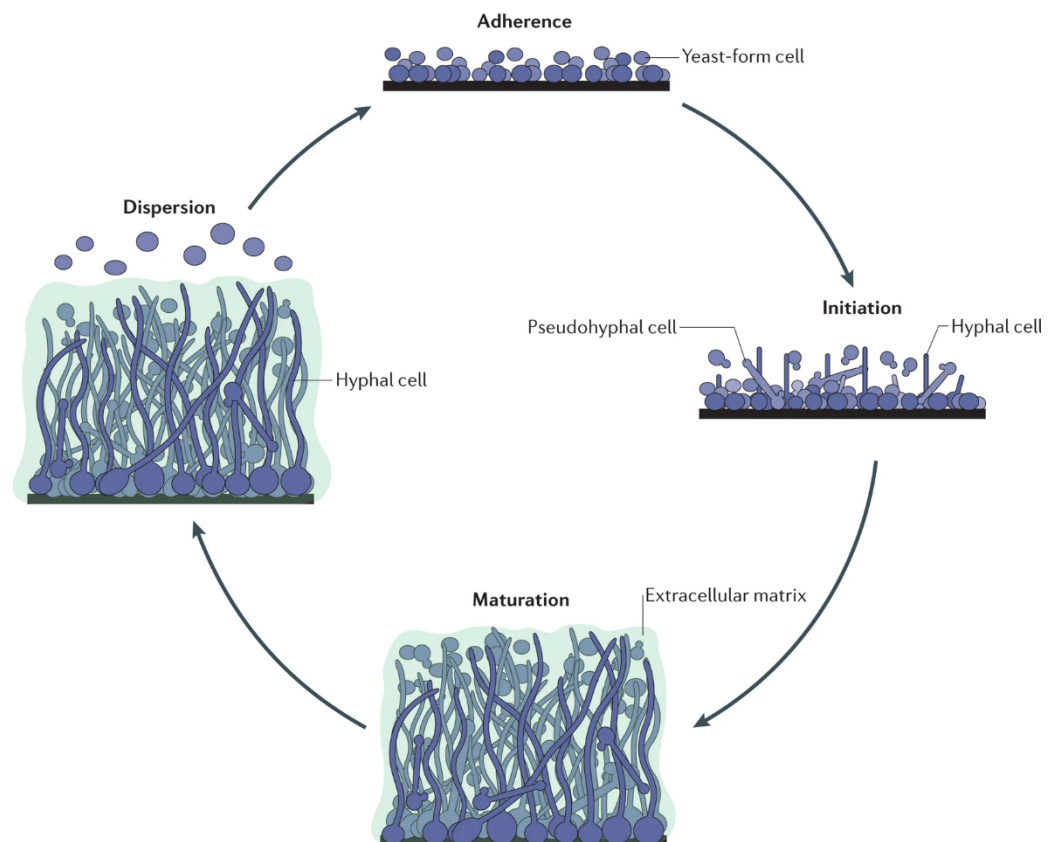


Figure 1.4: Stages of *C. albicans* biofilm development. Adapted from (Lohse et al., 2018).

C. albicans biofilms on vaginal mucosa have been observed previously by identification of significant fungal load, biofilm architecture and extra-cellular matrix using confocal and electron microscopy using a murine model (Figure 1.5) (Harriott et al., 2010a). Biofilms are a potential contributing factor to increased virulence and failed clinical treatment resulting in persistence of symptoms and recurrence of VVC. Despite the identification of *Candida* biofilms on vaginal mucosa, the predicted therapeutic challenge this presents in VVC is still disputed. Other studies deny the presence of vaginal biofilms, and instead suggest other factors such as germ tube formation and polymicrobial tissue invasion as a more critical feature of VVC (Swidsinski et al., 2018, Sobel, 2015a).

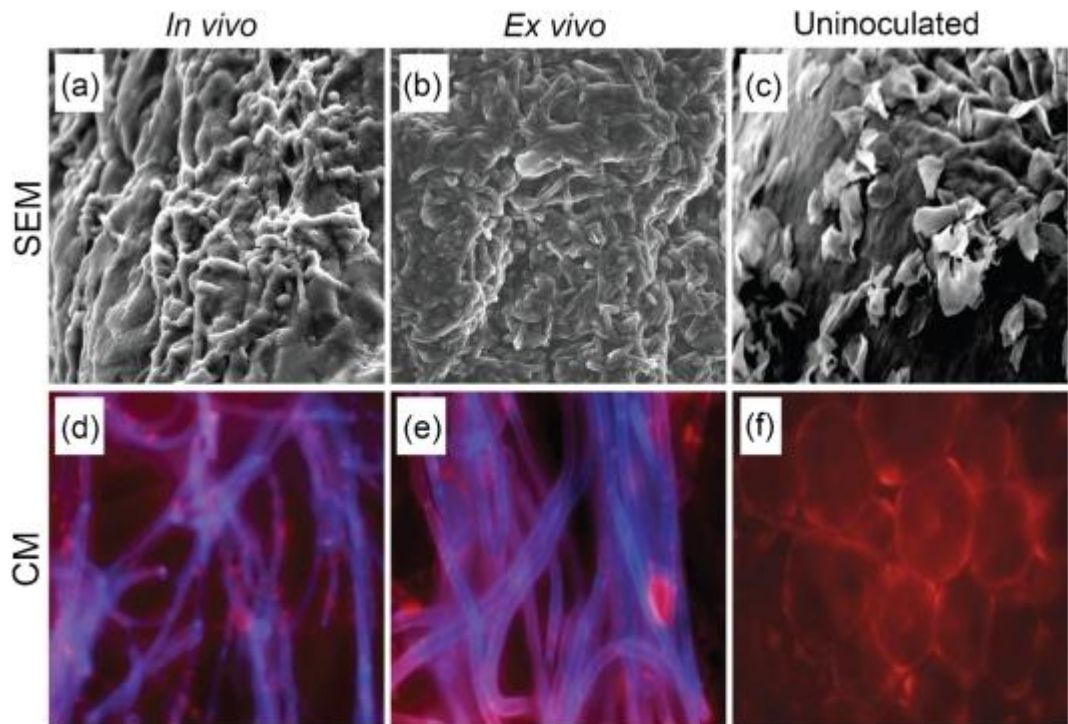


Figure 1.5: *C. albicans* biofilm formation on vaginal mucosa. *C. albicans* biofilm formation on murine vaginal mucosa in both an *in vivo* model (VVC induced in mice then vaginal tissue excised) and an *ex vivo* model (Mouse vaginal tissue excised before introduction of *C. albicans*). Taken from (Harriott et al., 2010b).

Through extensive research and clinical studies, it is well accepted that BV results from a loss of protective vaginal lactobacilli with a concomitant increase in BV-associated bacteria such as *G. vaginalis* and *A. vaginae* (Machado et al., 2015). These bacteria then form a thick *G. vaginalis*-dominated biofilm adhered to vaginal epithelium, dissemination of which results in diagnostic “clue cell” isolation in vaginal secretions (Swidsinski et al., 2005). Currently, there are no large-scale studies visualising *Candida* biofilm formation on vaginal biopsies in women with either VVC or RVVC, such as those which were carried out to investigate *G. vaginalis* biofilm formation in BV. Additionally, there are currently no defined VVC or RVVC biofilm models to study the impact of *Candida* biofilm formation in the disease. Such studies could provide key knowledge of RVVC pathogenesis and resistance which could greatly improve how clinical treatment is provided.

1.3.2 Resistance

The frontline treatment for VVC is orally administered fungistatic fluconazole. Although this is often effective in alleviating symptoms, antifungal tolerance in *Candida* is reported and can cause failed treatment with the need for prolonged suppressive therapies (Sobel et al., 2004). *Candida* biofilm formation is closely linked to development of antifungal tolerance, particularly to the azole family of antifungals. One study observed a 5 to 8-fold higher tolerance to various azoles, AMB and flucytosine in 48h *C. albicans* biofilms compared to their planktonic counterparts (Hawser and Douglas, 1995). Azole tolerance is most common in long-term suppressive treatment regimens, such as those used to treat RVVC. Various mechanisms of antifungal resistance in *Candida* to different therapeutics have been described and are graphically summarised in Figure 1.5.

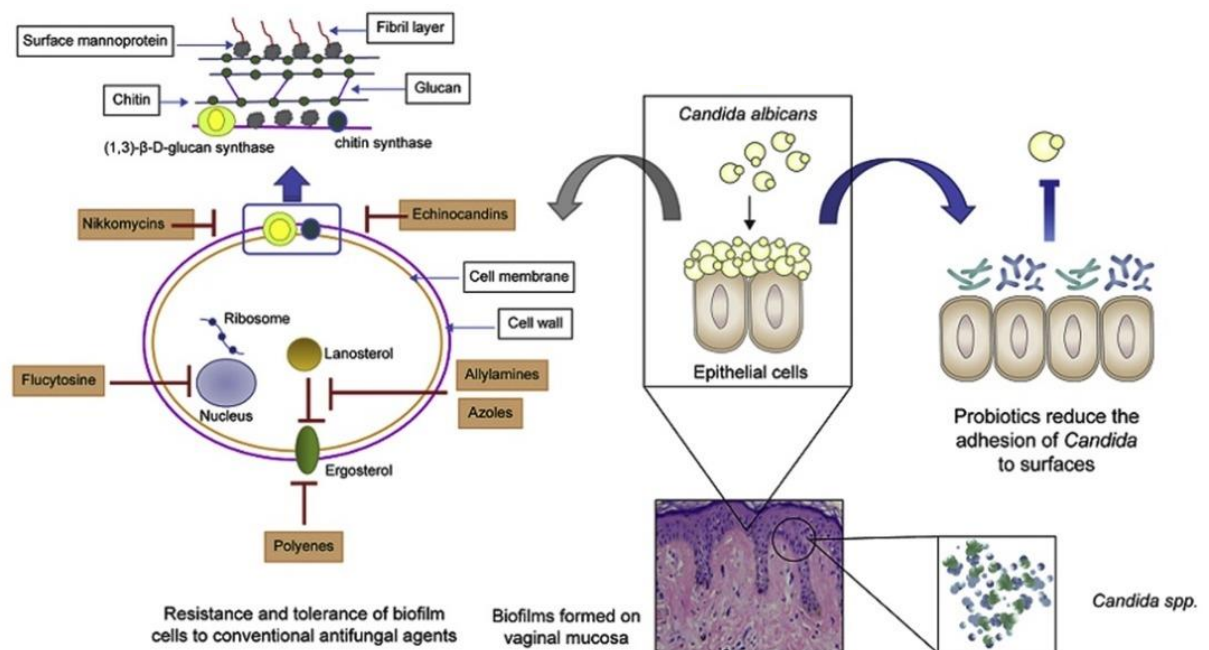


Figure 1.6: *Candida* colonisation of the vaginal mucosa and resistance mechanisms.

Azole resistance is achieved through increased expression of efflux pumps eliminating intracellular drug accumulation. Altered ergosterol content is thought to contribute to polyene resistance in *C. albicans*. Taken from (Rodriguez-Cerdeira et al., 2019).

Mechanisms of resistance to azoles in *Candida* include up-regulation of genes controlling efflux-pump activity such as Cerebellar Degeneration Related Protein 1 (CDR1) and Multi-drug Resistance 1 (MDR1) as well as mutations in the ERG11 target gene which reduce affinity and intracellular drug accumulation (Albertson et

al., 1996, White, 1997). Although acquired resistance is rare in *Candida* biofilms, intrinsic resistance to azole therapies is almost universal, making azoles completely ineffective for the treatment of biofilm-associated disease. This lack of efficacy against *Candida* biofilms is evident by the low cure rates of RVVC using suppressive azole therapies (Donders et al., 2008).

Other treatment options for azole-resistant isolates include the use of the polyenes, AMB and nystatin. Although AMB is effective against most systemic fungi including *Candida* species, it is associated with considerable toxicity and is rarely used in the treatment of VVC/RVVC. Resistance to AMB has been reported since the 1980s, with some *Candida* species including *C. lusitanae* and *C. guilliermondii* displaying intrinsic resistance (Dick et al., 1980, Kovacicova et al., 2001). More concerning is the development of AMB resistance by *C. albicans*, *C. glabrata* and *C. krusei* during treatment (White et al., 1998). Although the mechanism of AMB resistance in *Candida* is not fully defined, a recent study showed altered ergosterol content and mitochondrial function in AMB-resistant members of the *C. haemulonii* complex (Silva et al., 2020). Nystatin can be used as an alternative maintenance therapy to boric acid. Although studied to a lesser extent, due to the two drugs belonging to the polyene's family, resistance profiles to nystatin can be inferred from those to AMB.

It is important to note the potential presence of 'persister cells' in VVC/RVVC may contribute to failed clinical treatment. These small sub-populations of biofilms or biofilm aggregates provide protection to the cells within the population, similar to that seen in biofilms, allowing them to survive in the presence of antifungal intervention (Keren et al., 2004). Persister cells are commonly reported in bacterial biofilms however their presence in *C. albicans* biofilms has been observed (LaFleur et al., 2006). The presence of these free-floating biofilm aggregates may provide an explanation for failed clinical treatment in women where no clear biofilm is observed. Although it is currently being fully evaluated, no resistance to the novel drug, ibrexafungerp, has been reported to date.

Despite the well-accepted increased tolerance to antifungals as a result of biofilm formation, and the identification of *Candida* biofilms on vaginal mucosa, there are

currently no FDA-approved therapeutics specifically for women with biofilm-associated VVC/RVVC.

1.3.3 Clinical significance of non-*albicans* *Candida* species

As mentioned previously, NAC species are becoming more prevalent causative organisms in VVC worldwide. Although more often associated with milder symptoms than *C. albicans* VVC, NAC species often cause complicated VVC, with higher rates of recurrence due to increased antifungal resistance amongst these species (Deorukhkar et al., 2014). It is impossible to determine the true rates of NAC VVC due to underreporting, over-the-counter antifungal use, and misidentification. Under current diagnostic guidelines, following morphological identification of *Candida* using Gram stain and wet mount, identification of fungal species is carried out by observing germ tube formation. Positive germ tube formation results in a *C. albicans* identification, however both *Candida dubliniensis* and *Candida africana* also form germ tubes (Borman et al., 2013, Vilela et al., 2002). Diagnosis could be improved using chromogenic agars and newer molecular techniques which are more sensitive and specific with considerably faster output times and are being implemented to identify pathogens in STIs, however it is not yet common practice in VVC diagnosis (Sobel and Akins, 2015). Although it is commonly the case in a clinical setting, it is evidently important not to rely on microscopy, culture methods or PCR alone to identify *Candida* to species-level.

Risk factors associated with NAC-associated candidiasis include diabetes mellitus, older age (decreased oestrogen) and recent antifungal use. The pathogenesis of NAC VVC is more poorly understood than the more commonly studied *C. albicans* candidiasis. *C. glabrata* can employ an immune invasion strategy through survival within macrophage (Seider et al., 2014). Further, *C. glabrata* can suppress reactive oxygen species (ROS) production in the phagosome, preventing toxicity and suppressing proinflammatory responses (Kasper et al., 2015b). Although genetically similar to *C. albicans*, *C. dubliniensis* is considerably less pathogenic. This is likely in part due to the lack of genes encoding ALS3, SAP5 and SAP6 in *C. dubliniensis*, leading to reduced virulence and adhesion to vaginal epithelium (Moran et al., 2012).

Clinically, NAC VVC is more difficult to treat due to the antifungal resistant profiles of NAC species. Of particular clinical concern is the widespread resistance to frontline azole-family antifungals throughout NAC species. One study evaluating resistance profiles of *Candida* clinical isolates found 38%, 41%, 35%, 37% and 33% of *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. dubliniensis*, respectively, to be resistant to fluconazole, with similar profiles with itraconazole and ketoconazole (Deorukhkar et al., 2014). Further, MICs have been shown to increase by >4-fold following exposure to antifungals in vaginal NAC isolates (Richter et al., 2005). Notably, increased resistance to AMB and fluconazole has been observed in *C. glabrata* in low pH environments. Therefore, antifungal resistance could be exacerbated by the acidic vaginal environment during VVC (Danby et al., 2012). However, the effect of low environmental pH on the mode of action of azoles has not yet been investigated. This highlights the importance of careful consideration of the causative organism of VVC before clinical decisions for antifungal treatment can be made.

1.4 Treatment of VVC

VVC can be defined as either uncomplicated or complicated VVC. Uncomplicated VVC is defined as sporadic episodes of mild symptoms caused by *C. albicans* (Sobel et al., 1998). Whereas, complicated VVC is defined as severe infection, VVC caused by a NAC, *C. albicans* infection in an immunocompromised patient or development of RVVC (Sobel et al., 1998). Decisions for clinical treatment are determined by whether the patient has complicated or uncomplicated VVC (Pappas et al., 2009). For uncomplicated VVC the frontline therapeutic of choice for VVC is the azole-family of drugs given as either a topical cream or orally administered systemic treatment. A single dose of oral fluconazole is sufficient to treat sporadic *C. albicans* VVC in 80-90% of cases (Dovnik et al., 2015b).

Treatments for RVVC and VVC caused by NAC species are more complicated, requiring prolonged azole treatment as suppressive therapies and are often unsuccessful. As described in detail above, fluconazole treatments are completely ineffective against *C. albicans* biofilms and are a likely cause of failed clinical treatment of VVC and development of RVVC. In cases of complicated VVC such as

C. glabrata azole resistant VVC, daily treatment with boric acid or nystatin suppositories is recommended for two weeks. Alternatively, a topical treatment with 17% flucytosine can be administered alone or in combination with 3% AMB daily for two weeks. For treatment of RVVC, a 10–14 day maintenance therapy with topical or oral fluconazole is recommended followed by a weekly 150mg dose of fluconazole for 6 months. Despite known consequences of biofilm formation for therapeutic success, treatment options for women with azole-resistant isolates remain limited with the majority of recurrent cases required prolonged maintenance therapies which never fully cure symptoms of VVC/RVVC.

1.4.1 Current treatment options

Since their development in the 1980s, azole antifungals have been used to target ergosterol synthesis in the fungal cell wall. They target lanosterol 14 α -demethylase, encoded by ERG11, which disrupts ergosterol synthesis, inhibiting *Candida* growth and increasing susceptibility to host defences (Figure 1.7). No particular azole is thought to be more effective than another, additionally, it is unclear whether choice of oral or topical agents impacts clinical outcome (Dharmik et al., 2013, Whaley et al., 2016). Fluconazole is the most popular azole treatment for VVC due to its high cure rates against VVC, safety and availability both by prescription and over the counter. Fluconazole is also often prescribed as a maintenance therapy to treat RVVC taken as a weekly dose of 200mg for 2 months, followed by 200 mg every two weeks for 4 months, and 200 mg monthly for 6 months, depending on the patient's response to the treatment (Donders et al., 2008). Although this maintenance therapy is often sufficient to suppress recurrence of symptoms during treatment, it does not cure RVVC and is therefore often prescribed for years (Sobel et al., 2004). This long-term use and over the counter availability of fluconazole drives antifungal resistance and with no suitable FDA-approved alternatives leaves few options for the estimated 130 million women with RVVC each year.

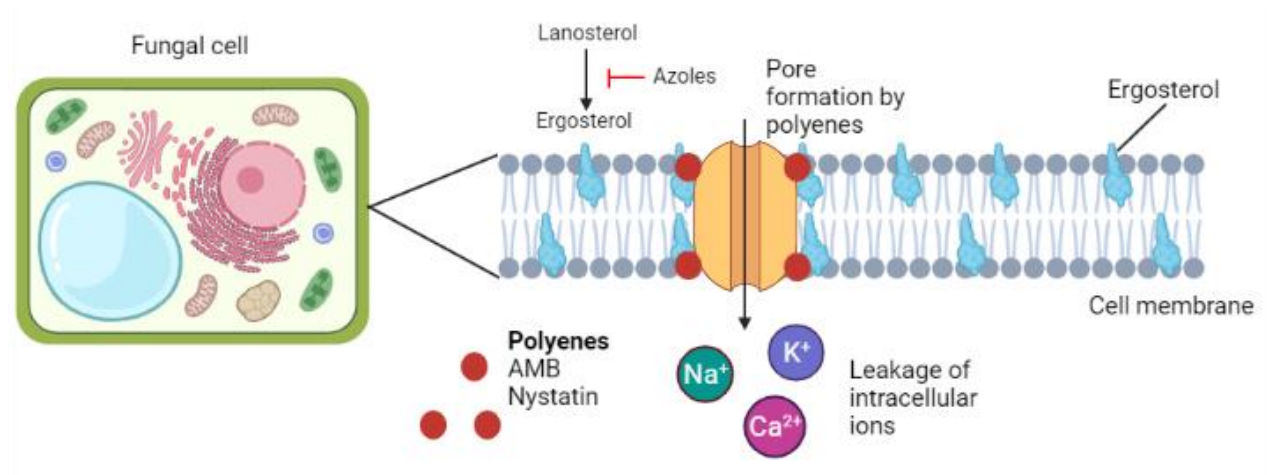


Figure 1.7: Mechanism of azole and polyene antifungal treatments. Azoles inhibit ergosterol synthesis from lanosterol resulting in fungal growth arrest. Polyenes, including amphotericin B (AMB) and nystatin, bind to ergosterol in the cell wall inducing pore-formation leading to disruption of the membrane potential, intracellular ion leakage and ultimately cell death. Created with Biorender.com.

Following confirmation of azole-resistance in VVC, daily treatment with 600mg of vaginally administered boric acid is routinely implemented for two weeks. However, boric acid has been shown to alleviate symptoms and fungal isolation in up to just 70% of cases (Sobel et al., 2003a). Boric acid is predominantly fungistatic and not fungicidal, although the mode of action is not fully elucidated. It is thought that its inhibitory effect may be due to its weakly acidic properties, however this is yet to be studied *in vitro*. One study identified significant reduction of CDR1 expression in *C. albicans* treated with boric acid, suggesting the drug may decrease efflux pump activity in *Candida* rendering it more susceptible to treatment with azoles (De Seta et al., 2009). This study also observed evidence of fungal growth arrest of *C. albicans*, similar to findings in *S. cerevisiae* which showed defects in cell budding and cell wall formation following boric acid treatment (Schmidt et al., 2010). Notably, despite its use as an alternative to antifungal treatment and claims of antifungal efficacy, very little data is available regarding the mechanisms of boric acid inhibition. Further, boric acid toxicity in humans has been documented when orally ingested or absorbed in to the bloodstream, indicating potential safety concerns (Moseman, 1994). An alternative to boric acid is the use of 17% flucytosine topical cream. Flucytosine has previously been shown to be effective in up to 90% of cases that failed boric acid and azole therapy (Sobel et al., 2003a). However, this study

was carried out on a small sample set (30 women) and there are very few studies investigating the efficacy of flucytosine against RVVC. Additionally, flucytosine is not commercially available and potential of acquired resistance in *Candida* is high, therefore it is rarely prescribed and not recommended for suppressive therapies (Horowitz, 1986).

The efficacy of therapeutics such as AMB and boric acid has been assessed for the treatment of RVVC. Both drugs when delivered intravaginally for 14-21 days were found to be effective in around 70% of patients (Phillips and gynecology, 2005) (Sobel et al., 2003b). Both drugs belong to the polyene family of antifungals and act by disruption of the fungal cell membrane through binding to ergosterol (Figure 1.7). This binding induces pore-formation resulting in intracellular ion leakage and disruption of the membrane potential leading to growth inhibition or cell death. AMB has also been shown to activate reactive oxygen species production inside the cell, increasing its fungicidal properties. The efficacy of oral fluconazole for the treatment of RVVC was directly compared with two-week treatments with another polyene, nystatin. Results demonstrated both drugs to be sufficient treatments for RVVC and concluded nystatin as a suitable alternative for the treatment of azole-resistant isolates (Fan et al., 2015). AMB is used in VVC therapy due to its wide antifungal spectrum and low resistance rates (Ci et al., 2018). Studies investigating the efficacy of AMB combination therapies against azole-resistant *C. glabrata* cases are promising with culture-negative results after up to 8 weeks of daily treatment with 100mg AMB and 1000mg flucytosine (White et al., 2001). Additionally, 50mg of AMB administered vaginally for two weeks was found to be effective in up to 70% of cases (Phillips, 2005).

1.4.2 Potential of pre/probiotic therapies

Probiotics involve the use of live microorganisms, whereas prebiotics are defined as “non-viable substrates that serve as nutrients for beneficial microorganisms harboured by the host, including administered probiotic strains and indigenous microorganisms” (Gibson et al., 2017). Lactoferrin, a naturally secreted antimicrobial peptide present in various bodily secretions including vaginal secretions and breast milk, is the most well-studied prebiotic due to its potent antibacterial and antifungal properties (Bertuccini et al., 2018, Russo et al., 2018, Liao et al., 2019). Moreover,

this key component of the innate immune system has been shown to inhibit *C. albicans* both alone and in cooperation with fluconazole (Wakabayashi et al., 1996, Lupetti et al., 2003). Both bovine and human lactoferrin bind to the plasma membrane of *C. albicans*, inducing apoptosis and can therefore be fungicidal in azole-resistant isolates (Andres et al., 2008). A recent clinical study assessed the efficacy of bovine lactoferrin in combination with probiotic *Lactobacillus* species *L. rhamnosus* ATCC SD5675 and *L. acidophilus* LMG S29159 to prevent recurrence of RVVC (Russo et al., 2019). Results from this study were promising with only 33.3% and 29.2% of treated women experiencing recurrence after 3 and 6 months, respectively compared with 91.7% and 100% of women in the placebo group at 3 and 6 months, respectively. More clinical studies assessing the efficacy of this combination prebiotic/probiotic treatment are required and may lead to improved therapeutics for the prevention of recurrent vaginitis in the future.

A recent extensive review of the potential of *Lactobacillus* probiotics for the treatment of VVC/RVVC concluded that despite promising studies, there remains insufficient evidence to support this therapeutic strategy at present (Falagas et al., 2006). Studies investigating the effectiveness of probiotic therapies over the last two decades have shown clear beneficial results for patients in the short-term. However, few studies have assessed the ability of probiotic to augment vaginal flora over longer periods of time (Jeng et al., 2020). Given the overall safety of the use of *Lactobacillus* probiotics and their potential efficacy in re-establishing eubiosis in the vagina *in vitro*, their use as a potential therapy merits further investigation. This would require large-scale, high-quality clinical trials recruiting women with VVC/RVVC to understand the effect of *Lactobacillus* probiotic therapy in the pathophysiological human environment.

The potential to treat RVVC through microbiome replacement therapy has been investigated, possibly through use of *Lactobacillus* probiotics. Probiotic therapy involves the administration of live microorganisms which directly result in a health benefit for the patient (Reid et al., 2003). Robust evidence exists showing *L. rhamnosus* GG successfully reduces disease duration of acute Rotavirus diarrhoea in children (Majamaa et al., 1995). Due to the diversity of the vaginal microbiome, it is difficult to determine which *Lactobacillus* species would be the most important to

replace with probiotic therapy. One study evaluated *L. plantarum* P17630 combined with the standard treatment of clotrimazole for 3 days and concluded a potential resolution of vaginal discomfort with the probiotic (De Seta et al., 2014). Additionally, the probiotic potential of *L. casei* var. *ramnosus* (now named *L. rhamnosus* GR-1) has been assessed. One clinical case study cured symptomatic RVVC for >6 months using *L. rhamnosus* GR-1 pessaries in a woman who had previously suffered 20 episodes of VVC in 30 months (Reid et al., 1994). Although not a vaginal commensal and only able to transiently colonise the vaginal tract, probiotic therapy with *L. rhamnosus* GR-1 may provide a scaffold for the re-colonisation of health-associated commensal *Lactobacillus* species.

Probiotic lactobacilli may also be used to augment treatment of vaginitis when used as an adjuvant therapy in addition to antifungal treatment. One study compared the treatment of VVC with 150mg oral fluconazole alone or 150mg oral fluconazole with bi-daily orally administered *L. rhamnosus* GR-1 and *L. reuteri* RC-14 in 55 Brazilian women (Martinez et al., 2009). This study observed a 28.2% reduction in *C. albicans* culture-positive patients with the probiotic adjuvant therapy compared with antifungal treatment alone. Similar observations were achieved when comparing fluconazole and fenticonazole treatments alone or combined with vaginally administered probiotic with *L. acidophilus*, *L. rhamnosus*, *Streptococcus thermophilus*, and *L. delbrueckii* subsp. *bulgaricus* (Kovachev and Vatcheva-Dobrevska, 2015). Use of a probiotic-antifungal adjuvant therapy may be more favourable as vaginal dysbiosis due to antifungal use can lead to onset of BV (Pawlaczyk et al., 2006), however this may be circumvented by maintenance of the microbiome by probiotic *Lactobacillus*.

1.4.3 Future therapies

A novel drug, approved for use to treat VVC in July 2021, Ibrexafungerp (previously SCY-078) may provide an alternative avenue for women experiencing failed treatment. Ibrexafungerp is the first of a novel “fungerp” class of triterpenoid glucan synthase inhibitor antifungals with distinct mode of action from echinocandins. Ibrexafungerp is administered orally and acts to destabilize the fungal cell wall through depletion of (1,3)- β -D-glucan polymers (Figure 1.8).

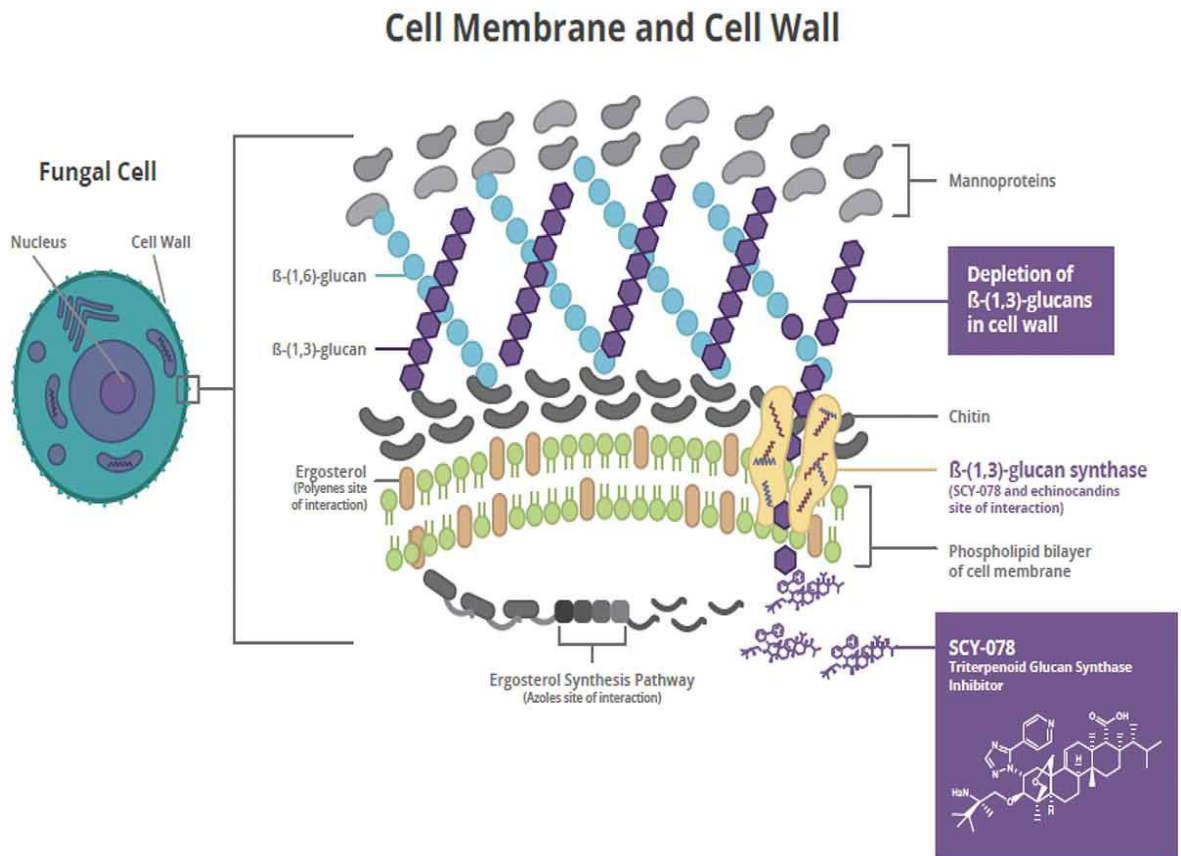


Figure 1.8: Mode of action of echinocandins and fungicidal antifungals on the yeast cell wall. Taken from (Aziz et al., 2020).

Resistance to echinocandins has been documented in various *Candida* species through the acquisition of point mutations in *FKS1* and *FKS2* genes. While resistance to ibrexafungerp has been shown in *C. glabrata* to be mediated by *FKS* mutations within the glucan synthase gene, the binding site for the drug is distinct from echinocandins resulting in lower rates of resistance (Jimenez-Ortigosa et al., 2017). Additionally, the activity of ibrexafungerp was shown to be minimally affected by the presence of echinocandin-resistance via introduction of *FKS* mutations in *Candida* species including *C. albicans*, *C. glabrata*, *C. krusei* and *C. dubliniensis* (Pfaffer et al., 2017). Increasing rates of resistance to both azoles and echinocandins in *C. glabrata* is of clinical concern (Ghannoum et al., 2019), however consistent results showing greater activity of ibrexafungerp against azole/echinocandin-resistant *Candida* species is promising for future treatment alternatives (Pfaffer et al., 2017). Further, the activity of fluconazole, micafungin and ibrexafungerp against *C. albicans* and *C. glabrata* were assessed at pH level of 7.0, 5.7 and 4.5 (Larkin et al., 2019). Unlike the other antifungals assessed,

ibrexafungerp displayed the most potent antifungal activity at a pH of 4.5, relevant to the vaginal environment.

Ibrexafungerp provides a novel class of antifungals with broad range of activity against *Candida* species and a distinct mode of action from azoles and echinocandins. Additionally, it has demonstrated good tolerability and low toxicity in recently completed phase 1 and phase 2 clinical trials (Akizawa et al., 2018). Ongoing phase 3 clinical trials will hopefully confirm the safety and efficacy of ibrexafungerp for the treatment of VVC. The approval of ibrexafungerp may provide an exciting alternative one-day treatment for women suffering from azole-resistant VVC/RVVC.

1.5 Next Generation Sequencing

Sequencing for long DNA reads using primer-extension and chain-termination methods, now commonly known as the Sanger sequencing method, was pioneered by the British biochemist, Frederick Sanger in 1977 (Sanger et al., 1977, Maxam and Gilbert, 1977). Following this valuable discovery, many landmarks in sequencing history were achieved including sequencing of the *Epstein-Barr* virus in 1984, Applied Biosystems marketing the first automated sequencer in 1987, and sequencing the genome of the nematode *Caenorhabditis elegans* in 1998 (Baer et al., 1984, Consortium, 1998). These breakthroughs paved the way to the completion of the Human Genome project to successfully sequence the human genome, which concluded in 2003. Although Sanger sequencing was highly accurate and resulted in the successful sequencing of the human genome, this chain-termination method was time-consuming and costly. It was estimated that the human genome project cost \$100 million in 2001. The introduction and development of Next Generation Sequencing (NGS) using pyrosequencing methods greatly reduced time and costs associated with genome sequencing to \$10,000 per genome by 2011 and just \$1,000 by 2015 (Pareek et al., 2011, Telenti et al., 2016). NGS sequencers include the Ion torrent, Roche 454, Illumina, and Nanopore platforms. Over the last 10 years, Illumina platforms have dominated the field of DNA sequencing due to their highly accurate read identification and accessibility to smaller labs (Bharti and

Grimm, 2021). Work in this thesis was carried out using the Illumina MiSeq platform with paired-end reads and the Nanopore MinION platform.

1.5.1 Illumina sequencing platforms

Illumina sequencing platforms utilise the sequence by synthesis method, reading one base at a time through a process known as bridge amplification. In this process, short DNA strands (up to around 350bp) are ligated at each end with adaptor sequences. These adaptor sequences are complementary to oligonucleotide strands attached to a solid support (flow cell). Adaptor sequences bind DNA for amplification to the flow cell, allowing bridges to form between sequences, where it is then subject to rounds of amplification which results in the formation of clonal clusters each containing 1000s of copies of each amplified DNA fragment. Each flow cell contains multiple channels containing millions of oligonucleotide sequences running in parallel, which is why techniques utilising this method are referred to as massively parallel sequencing. Fluorescently labelled nucleotides are then added to the flow cell and bind to their complementary bases on the amplified DNA fragments resulting in specific fluorescent emission detectable using sequential imaging of the flow cell.

Illumina sequencers have improved in throughput and accuracy with each generation, with Phred scores >40 (De Maio et al., 2019). Currently, the most widely used Illumina sequencer is the HiSeq platform. The MiSeq sequencer is more financially accessible for smaller labs with high accuracy, an output range of 540mb – 15gb, 1-25 million reads per run, and a maximum read length of 2x300 bp. The more powerful Illumina sequencers, NextSeq and NovaSeq are available for more extensive applications such as large whole-genome and whole-transcriptome sequencing. The 16S rRNA gene is around 1600bp long and is made up of 9 hypervariable regions (V1-V9) (Wang and Qian, 2009). Sequencing the more conserved regions is often utilised to determine higher-ranking taxa such as phylum or family, however regions with higher levels of variability are used to identify bacteria to genus or species-level (Kim et al., 2011). As Illumina sequencers only amplify a subsection of the 16S rRNA gene, careful consideration of the region of interest is required during experimental design. Additionally, studies sequencing mock communities have identified the introduction of bias and disproportionate taxa

based on the chosen amplified region (Yu et al., 2008, Yang et al., 2016). Although, increasing read length could circumvent this limitation of Illumina sequencing, this can greatly increase error rates (Tan et al., 2019).

1.5.2 Oxford Nanopore MinION platform

Nanopore sequencing using Oxford Nanopore Technologies (ONT) MinION sequencer is the latest long-read, single-molecule sequencing platform to be developed. The proposed method of Nanopore sequencing in the late 1990s would push a single strand of long DNA through a protein nanopore, stabilised in a thin, electrically resistant polymer membrane (Kasianowicz et al., 1996). An electric current would then be passed across the membrane and the ion current changes introduced by the passing nucleotides are used to determine each base in real time. However, it would be over 15 years before a collaboration between Professor Hagan Bayley and Oxford Nanopore would lead to the first benchtop sequencer using this method, the MinION, being commercialised (Jain et al., 2016). Each MinION flow cell has 2,048 individual nanopores embedded in separate membranes, of which 512 are selected for analysis. Despite its ultra-long reads, the MinION has lower accuracy than Illumina sequencers with Phred scores often <10 (Laver et al., 2015). According to the ONT, the average accuracy of the latest R10.3 flow cell is >99.9% with an output of up to 50Gb (Liu et al., 2021). Error rates are dictated by achieving the correct translocation speed of the DNA strand through the pore and have been shown to increase in longer sequencing runs (Meller et al., 2000). ONT has also introduced the GridION (100Gb output) and PromethION (6Tb output) sequencers which can run multiple flow-cells in tandem allowing for larger sequencing runs.

The MinION is the most widely used of ONTs sequencing platforms, being used in many genomic and transcriptomic studies (Kerkhof et al., 2017, Benitez-Paez and Sanz, 2017, Sessegolo et al., 2019). The advantages of the MinION are its portability, high throughput library preparation and relatively low start-up costs. Additionally, the ultra-long read capacity allows for taxa identification by amplification of the whole 16S rRNA gene, eliminating the risk of bias introduced through region selection. Another advantage of the ONT platforms is the ability to analyse data from the beginning of the run and as sequencing progresses, allowing for considerably reduced analysis time compared with Illumina sequencers. Finally,

ONT research is largely community driven with the “Nanopore community” being developed to allow users to troubleshoot and access sequencing and analysis protocols. However, the biggest short fall is the MinION has a typical accuracy of 95% compared with 99.9% of Illumina.

Nanopore sequencing, although rapidly developing, is still in its infancy and one of the major pitfalls is the current lack of bioinformatic tools for analysis of sequencing data produced by this method (Santos et al., 2020). The most common and user-friendly tool is the ONT developed data analysis web service, EPI2ME. This tool is available to ONT customers and provides workflows for analysis of nanopore sequencing data from barcoding to filtering reads and taxonomic assignment using BLAST to the NCBI database. However, the parameters for filtering reads, trimming and alignment are set by the web tool and are not adjustable, limiting the user’s analysis. The most prominent drawback encountered by ONT users is the lack of compatibility of the taxonomic assignment output with other bioinformatic analysis tools for downstream analysis. The most commonly used databases for taxonomic identification include Greengenes (McDonald et al., 2012), SILVA (Quast et al., 2013), RDP (Cole et al., 2014) and NCBI (Federhen, 2012), however these were developed for compatibility with Illumina data outputs and are not compatible with Nanopore outputs. Although the MinION sequences the full 16S rRNA region, allowing for accurate microbial identification to species-level, it is important to note its analytical limitations (Santos et al., 2020). Significant improvements to the error rates have been made since the development of the MinION sequencer, however more sophisticated bioinformatic tools designed for compatibility with Nanopore sequencing outputs are required to fully exploit the capabilities of this novel technology.

1.6 Summary and Aims

The literature reviewed in this introduction outlines our current understanding of VVC/RVVC onset, epidemiology, microbiological factors, and treatment. Despite exceptionally high annual case numbers globally, VVC/RVVC remains a significant health burden with debilitating recurrent cases severely impacting the mental health of affected women. This review highlights caveats in our knowledge of RVVC. Specifically, the importance and clinical consequence of *Candida* biofilm formation on vaginal mucosa and increased antifungal tolerance during RVVC remains largely understudied in the field of women's health. Additionally, relatively little is known about the role of the microbial populations and potentially important bacterial interactions taking place during RVVC. Finally, despite high levels of recurrence and failed clinical treatment, alternative treatment options for women with azole-resistant isolates remains limited to prolonged suppressive therapies which are often unsuccessful in curing disease. A greater understanding of VVC/RVVC could greatly reduce annual cases and lead to the development of appropriate therapies for women with recurrent, azole-resistant isolates.

It is hypothesised that clinical samples from patient with RVVC will display a biofilm phenotype with the propensity for antifungal resistance. Additionally, based on previous studies, the microbiome of RVVC is expected to be somewhat similar to healthy patients, however subtle differences could result in altered microbial interactions during RVVC. The aims of this study were therefore to:

- Gain insights into the vaginal environment during health and RVVC by assessing a panel of RVVC clinical samples and observe potential sources of failed clinical treatment.
- Investigate variations in microbial populations in health and RVVC with respect to patient treatments and behaviours.
- Observe interkingdom interactions between *Candida* and health-associated *Lactobacillus* species which may influence disease.

2 Bibliometric analysis

2.1 Introduction

Antagonism between *Candida* and *Lactobacillus* species has been documented for decades, primarily in the study of oral and gastrointestinal candidiasis (Kohler et al., 2012, Hasslof et al., 2010). Despite knowledge that *Lactobacillus* inhibits *Candida* adhesion and hyphal morphogenesis, *Lactobacillus* probiotics are not commonly used as therapeutics in antifungal-resistant vaginitis. Further, the potential formation of *Candida* biofilms at mucosal sites, and how this may impact interactions between *Lactobacillus* and the fungus, remains largely understudied, particularly in vaginal candidiasis. Within the scientific community, to assess understanding of a particular field of research and identify potential caveats in current knowledge, an extensive literature review of the subject area is carried out (Snyder, 2019). However, as the number of publications in each field continues to expand and datasets become larger through increased use of omics technology, it has become increasingly difficult to evaluate entire fields of research in a meaningful way. Although originally introduced in 1987, bibliometric analysis to perform quantitative assessment of entire fields of scientific literature is only recently becoming more widely used (Broadus, 1987). The ability of this technology to manage and assess large volumes of publications may provide a solution for large-scale *in silico* analysis, which previously would not have been possible.

Although bibliometric analysis provides a unique tool for managing the expanding pool of scientific literature, it requires an expertise in programming language software such as R or Python to perform. This is because bibliometric analysis requires various steps of analyses and mapping using this software (Guler et al., 2016). Many researchers are not proficient in such software packages, the majority of which also require commercial licenses. The complexity of the process ultimately limits the potential of bibliometric analysis for most researchers simply due to a lack of programming knowledge and accessibility. However, a recent novel tool developed in R to carry out comprehensive bibliometric analysis through a web application has been developed (Aria and Cuccurullo, 2017). This open-source Biblioshiny[®] tool allows researchers to import files containing research items retrieved from databases such as Scopus and Web of Science. Researchers can then perform comprehensive bibliometric analyses using the web application. As it is programmed in R, Biblioshiny[®] can perform various graphical techniques and is

easily upgraded and modified to suit developing bibliometric analysis. The development of this resource has greatly increased the availability and potential of bibliometric analysis without the need for extensive knowledge of programming language software.

Hypotheses and aims

In the age of increasingly concerning widespread antimicrobial resistance and more research focussing on alternative treatment therapies, the potential of *Lactobacillus* species to treat biofilm-associated candidiasis infections may become more apparent. Due to controversy regarding the presence of *Candida* biofilms in the vagina, it is expected that few research items will be retrieved in this area of research. This chapter was written as a mitigation strategy during successive lockdowns and lab closures due to the COVID-19 pandemic. Therefore, this chapter aimed to:

- Utilise bibliometric analysis using the Biblioshiny® web tool to observe the fields of scientific literature where interkingdom interactions between *Candida* and *Lactobacillus* are most widely recognised.
- Investigate where in the literature the impact of *Candida* biofilm formation is appreciated.
- Finally, this chapter aimed to investigate these themes with respect to vaginitis infection. It is expected that *Candida* and *Lactobacillus* research will primarily be focussed on oral health and the treatment of oral candidiasis.

2.2 Materials and Methods

To produce data for this chapter, the online web application, Biblioshiny® was used (Aria and Cuccurullo, 2017). This relatively novel software provides an open-source tool allowing researchers to perform comprehensive science mapping analysis of specific fields of scientific literature without requiring an extensive knowledge of programming language software such as R.

2.2.1 Data collection

Data for this chapter was collected from the Clarivate Analytics Web of Science™ online database by utilising different search terms which are defined in Figure 2.1. The initial search for metadata relating to “*Candida*” and “*Lactobacillus*” retrieved 2,330 articles from the Web of Science Core Collections™ database. An additional search using the search terms “*Candida*”, “*Lactobacillus*” and “Biofilm” was also carried out. This search retrieved 188 items. Finally, a search for metadata relating to “*Candida*”, “*Lactobacillus*”, “Biofilm” and “vaginitis” was carried out, however this only retrieved 8 items. Metadata relating to each search were then exported in batches of 500 before being merged in to one master file to be uploaded to the Biblioshiny® web application for analysis.

2.2.2 Bibliometrics analysis

To carry out bibliometrics analysis on the retrieved metadata, the Biblioshiny® web application available through the *Bibliometrix*® package (Version 3.0.0), loaded via R-Studio® (Version 1.2.5042) was utilised. Using the Biblioshiny® application, search results were refined to include only articles published between 1990-2020. Search results were also filtered to exclude corrections, editorials, letters, notes and reviews. This resulted in a total of 1,860 and 155 research items relating to the “*Candida*” and “*Lactobacillus*” search and the “*Candida*”, “*Lactobacillus*” and “Biofilm” search, respectively. The online web application was then used for data visualisation relating to annual scientific production, relevant sources, author contributions, common keywords, and collaborative links for each search. An illustration of the bibliometric analysis workflow can be seen in Figure 2.1.

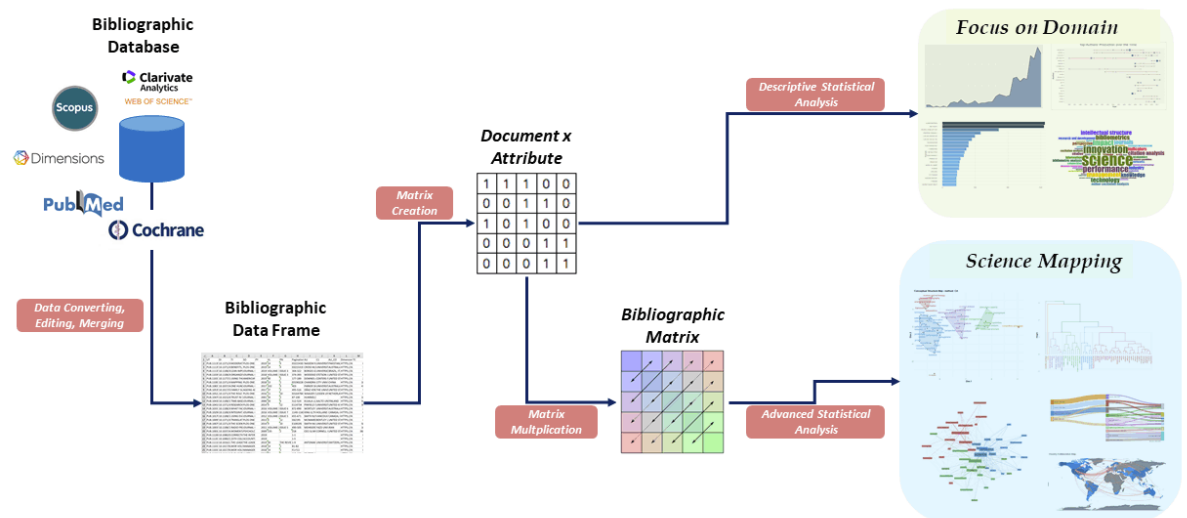


Figure 2.1: Comprehensive science mapping workflow provided by the Biblioshiny® web application (Aria and Cuccurullo, 2017). Bibliometric data is collected, formatted, and exported from an online database. Data is then uploaded to a bibliometric software tool for analysis and normalisation before data can be visualised and interpreted.

2.3 Results

Search terms were designed to investigate the relevance of interkingdom interactions between *Candida* and *Lactobacillus* and to determine which fields of research these interactions are currently being appreciated. Search terms used and the number of metadata retrieved from each can be found in Table 2.1.

Table 2.1: Search terms used to obtain research articles from the Web of Science Core Collection™ database.

WoS search terms	Articles	Filtered articles
"Candida" and "Lactobacillus"	2,330	1,860
"Candida", "Lactobacillus" and "Biofilm"	188	155
"Candida", "Lactobacillus", "Biofilm" and "Vaginitis"	8	3 (5 reviews)

Analysis of the annual growth rate of *Candida* and *Lactobacillus* research observed a steady increase in the number of research articles produced from 1990-2020 (Figure 2.2). Annual production in the field was relatively low throughout the 1990s with an average of 22 research articles published each year, increasing towards the end of the decade. This average annual growth rate from 1990-2004 was 8.36%. A notable increase of interest in the field can be observed from 2004 onwards, with an average of 89 annual publications between 2004-2020. An average annual growth rate of 7.7% was observed for this period. A peak in annual publications is seen in 2019 with 145 articles, however, given the increasing trend this will likely be surpassed in coming years.

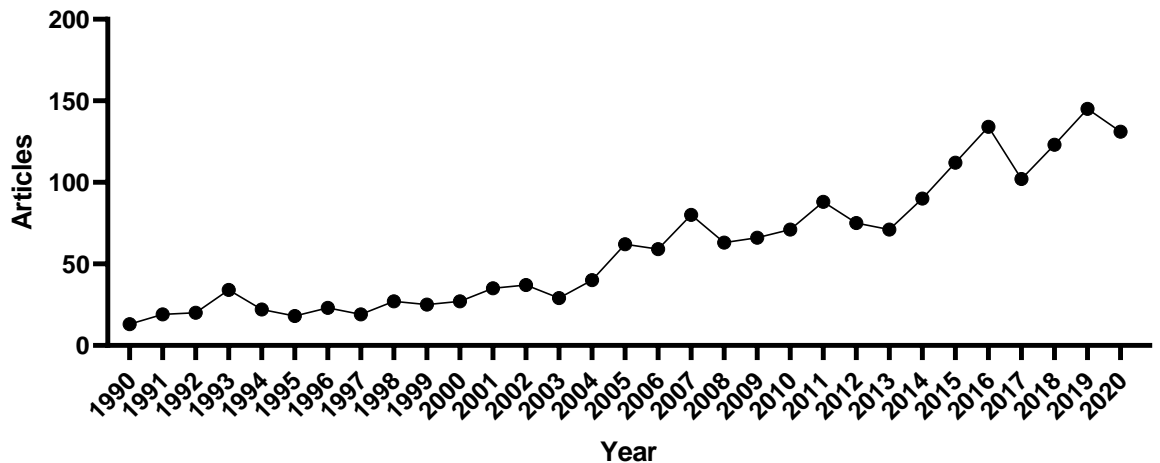


Figure 2.2: Annual growth of scientific articles published in *Candida* and *Lactobacillus* research. Number of articles published in the field of *Candida* and *Lactobacillus* research between 1990-2020.

To further assess levels of interest in *Candida* and *Lactobacillus* research, the total and average number of citations each year were observed (Table 2.2). Despite there being relatively few publications per year in the 1990s, total citations each year are relatively high with a peak of 52 citations in 1998. Total numbers of citations remained at this level until 2010, where a steep drop is noted with <20 total citations from 2015-2020. When average citations per article per year is observed, many of the articles published in the 1990s were receiving very few publications with an average of between 1-2 citations per article. Interestingly, despite there being fewer citations overall in later years, each article was receiving between 2-4 citations throughout the 2000s. This could suggest that fewer authors are publishing in the area as it becomes of less global interest. However, it may also reflect the sources of these articles becoming more stringent and some papers which may have been published in the past decade would not be accepted by present peer-review procedures.

Table 2.2: Annual scientific production and citations in the years between 1990-2020 in *Candida* and *Lactobacillus* research.

Year	No. of articles	Total citations	Average citations per article per year
1990	13	22.15	0.71
1991	19	32.79	1.09
1992	20	25.45	0.88
1993	34	34.15	1.22
1994	22	21.64	0.80
1995	18	38.28	1.47
1996	23	50.09	2.00
1997	19	39.32	1.64
1998	27	52.30	2.27
1999	25	47.24	2.15
2000	27	33.52	1.60
2001	35	32.46	1.62
2002	37	58.03	3.05
2003	29	49.62	2.76
2004	40	37.70	2.22
2005	62	51.65	3.23
2006	59	36.53	2.44
2007	80	37.13	2.65
2008	63	41.43	3.19
2009	66	40.74	3.40
2010	71	37.45	3.40
2011	88	24.52	2.45
2012	75	24.63	2.74
2013	71	34.51	4.31
2014	90	27.17	3.88
2015	112	18.99	3.17
2016	134	13.14	2.63
2017	102	15.66	3.91
2018	123	7.71	2.57
2019	145	4.71	2.36
2020	131	1.15	1.15

To investigate the most relevant sources of *Candida* and *Lactobacillus* research, the top ten scientific journals publishing relevant research were plotted along with their dynamics between 1990-2020 (Figure 2.3). This data also allowed for an insight into which fields of literature *Candida* and *Lactobacillus* research are being published. The most published in scientific journal was the International Journal of Food Microbiology, the source of 24% of total publications (Figure 2.3A). Although there was a sharp rise in publications from this journal from 1998-2004, annual production has since continued to drop steadily. Despite Food Microbiology being the second most relevant source, dynamics show a significant decrease in publications from 2012 (Figure 2.3B). Emerging sources such as PLOS One and LWT-Food science and Technology have increased rapidly since 2010 and will likely become the most relevant sources in the coming decade. It is clear from this data that the most relevant sources of *Candida* and *Lactobacillus* research publish in the field of microbiology with particular emphasis on the food industry.

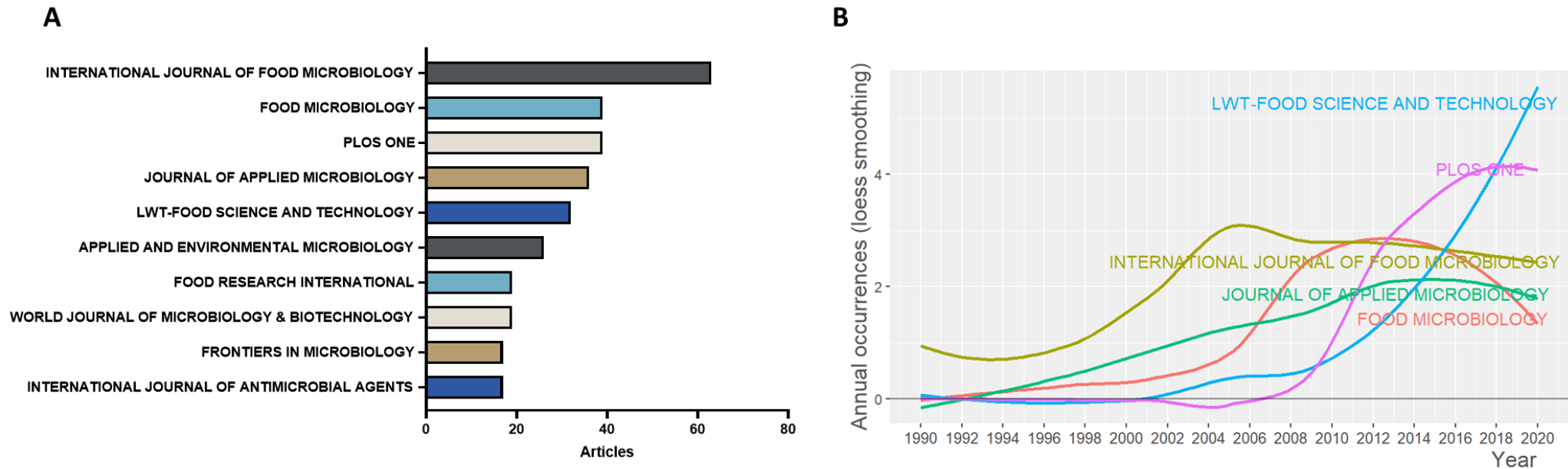


Figure 2.3: Most relevant sources of *Candida* and *Lactobacillus* research

Top 10 most relevant sources of scientific literature and number of articles published in *Candida* and *Lactobacillus* research between 1990-2020 (A). Dynamics of the top 5 most relevant sources in the field between 1990-2020 (B).

To further investigate relevant scientific fields within *Candida* and *Lactobacillus* research, the top 10 most relevant authors publishing between 1990-2020 based on numbers of published articles and their collaborative links were observed (Figure 2.4). A relatively small range between the most relevant authors was observed with between 10-13 published articles (Figure 2.4A). One of the top publishing authors, Jorge A. O. C., was found to have strong collaborative links with other relevant authors, Junqueira J. C. and Rossoni R.D. (Figure 2.4B). However, no collaborative links with other authors in the field were identified for the other most published author, Meurman J. H. Additionally, other identified relevant authors including Bellen G., Donders G. G. G. and Vitali B. were found to have close collaborative links.

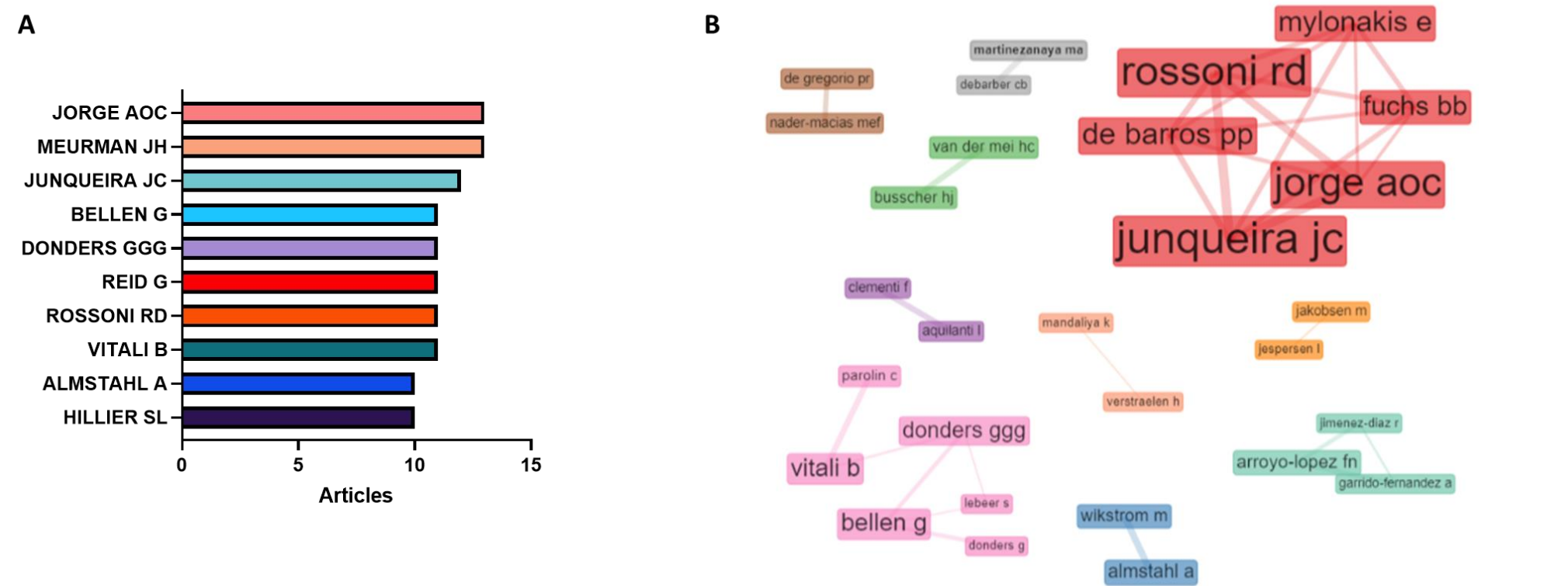


Figure 2.4: Relevant authors and collaborative links in *Candida* and *Lactobacillus* research

Relevant authors based on number of published articles in the field between 1990-2020 (A). Network of collaborations between the most published authors (B). Node size is weighted by author contribution; Closed networks of one colour represent closely linked collaborative groups. Isolated nodes were removed.

To investigate global contribution to the field of *Candida* and *Lactobacillus* research, scientific production was observed with respect to the corresponding author's home country (Figure 2.5). Countries identified with a larger contribution to the field are indicated by dark blue colouration and primarily include countries in North America, Europe and eastern Asia. Countries with a lower contribution are shown in light blue and include countries in Africa, South America, and central Asia. Finally, countries with a grey colour were found to have no publications relative to *Candida* and *Lactobacillus* research.

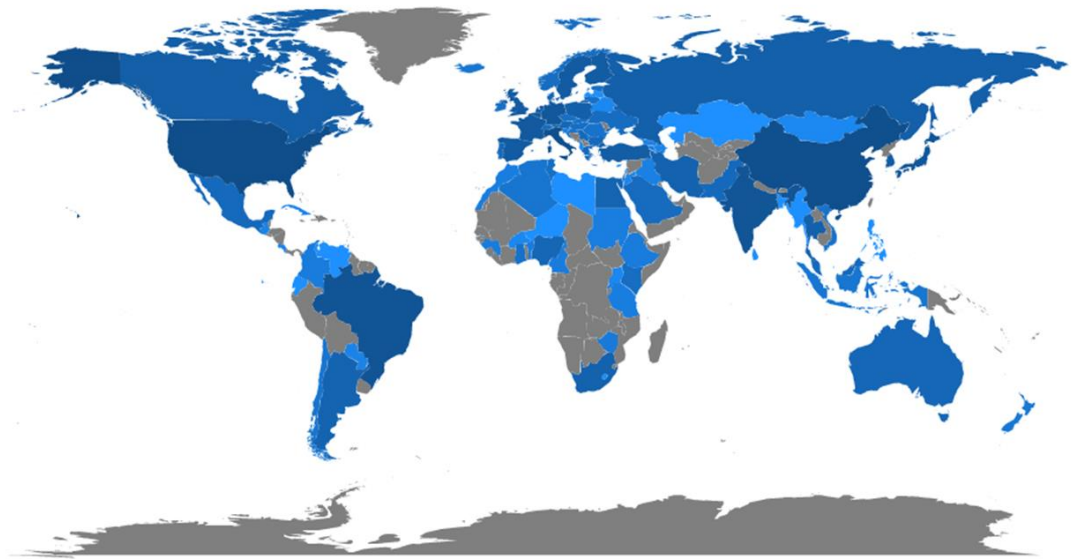


Figure 2.5: Scientific production by country of *Candida* and *Lactobacillus* research. Scientific contribution by country indicated by dark blue (large contribution), light blue (small contribution) and grey (no contribution) to *Candida* and *Lactobacillus* research between 1990-2020.

Further to the previous analysis, relevance of each country by scientific contribution to the field was assessed. (Figure 2.6). The highest contributing countries were found to be China and the USA, closely followed by Italy with between 139-169 publications (Figure 2.6A). All countries were found to primarily publish as single country publications (SCP). The USA had the most multiple country publications (MCP) with 19% of total publications involving collaboration with another country. Further, the relevance of these countries to the field was measured by the number of citations per article (Figure 2.6B). The USA and Italy had the most total citations with >5000 citations each. However, despite having the highest total contribution to the field, China had significantly fewer citations than the USA and Italy with 2999 total citations and the lowest average citations per article at 18. Interestingly, countries with a much smaller total contribution such as Belgium, closely followed by Germany and Sweden were found to have the highest numbers of citations per article with an average of 44 citations per publication.

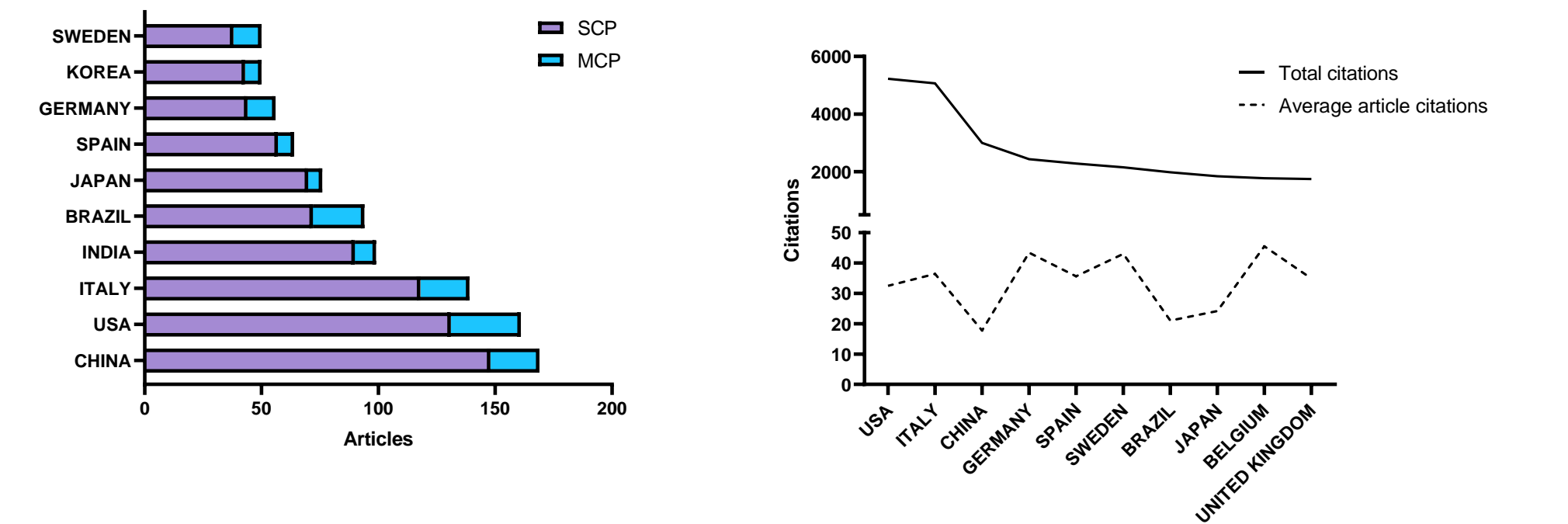


Figure 2.6: Relevance of scientific contribution by country.
Number of articles published by the corresponding author’s country (A). Split bars represent single country publications (SCP) and multiple country publications (MCP). Total and average number of citations from the highest published countries between 1990-2020 (B).

To investigate the content and common themes of articles published in the field of *Candida* and *Lactobacillus* research, the most common author's keywords and their correlations were observed (Figure 2.7). Key themes are correlated by colour and plotted based on their appearance in the literature. The size and overlap of bubbles in this plot represent overlap of themes within the field. Probiotics was identified as a 'basic' or widespread theme in the bottom right quadrant of Figure 2.7A. Keywords associated with the theme of probiotics included "lactic-acid bacteria", "fermentation" and "yeast" (Figure 2.7B). The size and centrality of nodes represents their frequency of co-occurrence in the literature. The top right quadrant, indicating 'motor', or re-occurring themes, identified "lactic-acid bacteria" and "*Candida albicans*" as themes in the literature. Keywords associated with "lactic-acid bacteria" include "sourdough" and "metabolism"; Keywords such as "saliva", "lactobacilli" and "dental caries" were identified as important in the theme of "*Candida albicans*". One theme identified as either emerging or declining was "saliva" which was associated with keywords such as "*Lactobacillus*", "*Candida albicans*" and "candidiasis". Finally, the theme of "bacteria" was identified as a central theme in *Candida* and *Lactobacillus* research and is associated with keywords such as "cholesterol" and "probiotic".

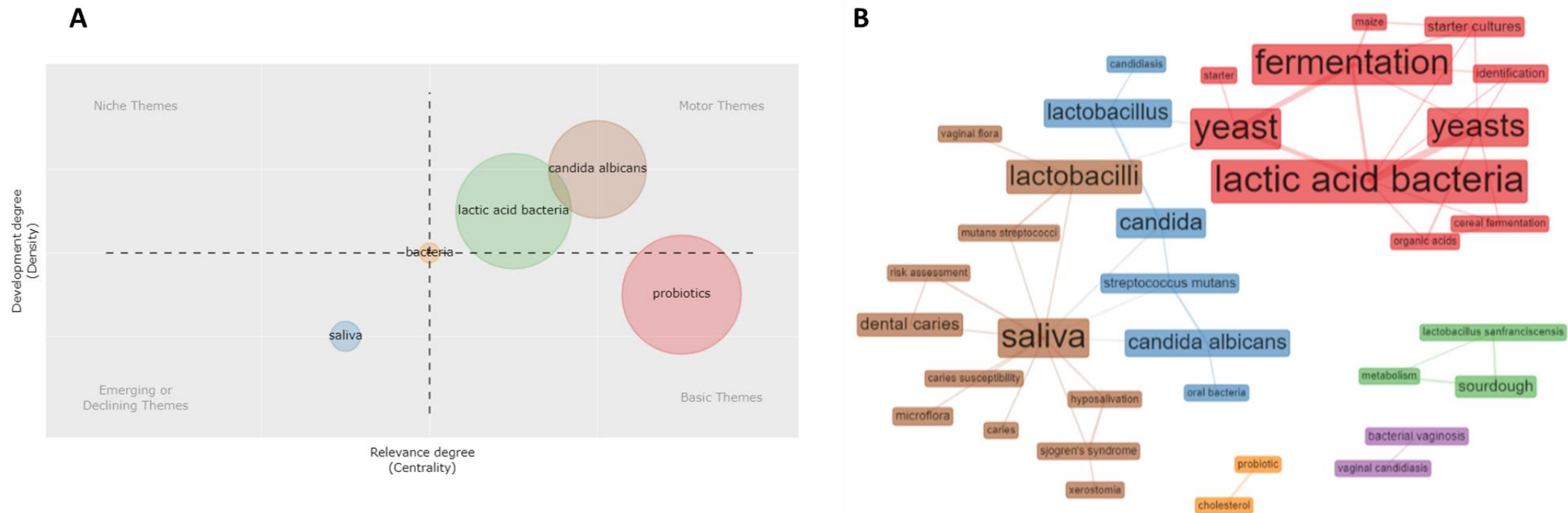


Figure 2.7: Most frequent keywords and their correlations in *Candida* and *Lactobacillus* research.

Thematic map illustrating the relevance of common keywords in *Candida* and *Lactobacillus* research (A). Co-occurrence network of the most common author's keywords used in the field (B). Node clustering and size is weighted by co-occurrence and frequency of keywords, respectively. Isolated nodes were removed.

Next, a second bibliometric search was conducted using the search terms “*Candida*”, “*Lactobacillus*” and “Biofilm”. This allowed for further insight into which fields of research *Candida* and *Lactobacillus* research is focused as well as to determine whether the impact of biofilms is appreciated in scientific literature. This search returned only 188 metadata items, 155 after filtering, significantly fewer articles than were found in the previous search (Table 2.1).

When assessing the annual scientific production of *Candida* and *Lactobacillus* biofilm research between 1990-2020, it was clear that there are much fewer publications in this field with no more than 25 articles in any given year (Figure 2.8). In-fact, no articles were published in this area pre-1999. Less than 10 articles were published in this area throughout the 2000s, however the past decade has shown a notable rise in publications since 2014. This increase in interest in the field is evident with average annual growth rate over the period from 1999-2020 of 17.42%. A peak in annual scientific production is seen in 2016 with 25 articles, however interest remained relatively high in the years that followed.

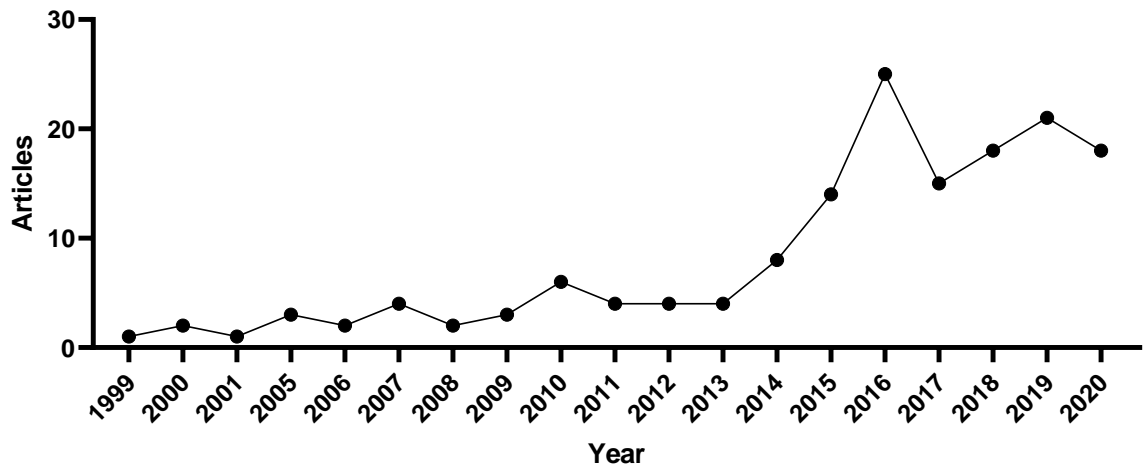


Figure 2.8: Annual growth of scientific articles published in *Candida* and *Lactobacillus* biofilm research. Number of articles published in the field of *Candida* and *Lactobacillus* biofilm research between 1990-2020.

Although relatively few research items were published pre-2014, the impact of these research items is strikingly high when observing annual citation statistics (Table 2.3). There were between 0-3 annual publications throughout the 2000s, despite this, there is an average of 23 total citations each year. Although, it is important to note that, because these articles were published almost two decades ago, the average citations per year remains relatively low. Since 2010, the increase in interest in the field is apparent with more publications each year. This is concurrent with a notable increase in the total number of citations per year and indeed the average citations per article per year. An impressive peak in research interest can be seen in 2010 with 92 total citations over 6 articles, resulting in an average of 8 citations per article. As the number of research items published annually rises, the total number of citations remains steadily high with an average of 17 citations between 2015-2019.

Table 2.3: Annual scientific production and citations in the years between 1999-2020 in *Candida* and *Lactobacillus* biofilm research.

Year	No. of articles	Total citations	Average citations per article per year
1999	1	26.00	1.18
2000	2	21.00	1.00
2001	1	18.00	0.90
2002	0	0.00	0.00
2003	0	0.00	0.00
2004	0	0.00	0.00
2005	3	63.67	3.98
2006	2	54.50	3.63
2007	4	30.50	2.18
2008	2	9.00	0.69
2009	3	33.67	2.81
2010	6	91.67	8.33
2011	4	18.50	1.85
2012	4	30.50	3.39
2013	4	16.00	2.00
2014	8	54.50	7.79
2015	14	23.43	3.90
2016	25	19.92	3.98
2017	15	11.73	2.93
2018	18	9.72	3.24
2019	21	6.24	3.12
2020	18	0.50	0.50

The most relevant sources of scientific literature and their dynamics in the field of *Candida* and *Lactobacillus* biofilms were observed (Figure 2.9). This allowed for insight into which fields of research articles are being published. Unlike the previous search, the most relevant sources of scientific literature were found to be related primarily to medicine and oral microbiology (Figure 2.9A). Although PLOS One was found to be the most relevant journal, source dynamics show a sharp fall in the number of articles published here since 2015 (Figure 2.9B). The second most published journal, Archives of Oral Biology, displayed a steady rise in publications since 2011. Other journal including the Journal of Applied Microbiology, Journal of Dentistry and Microbial Pathogenesis have shown a steady increase in the number of publications since 2011, illustrating increased interest in the field.

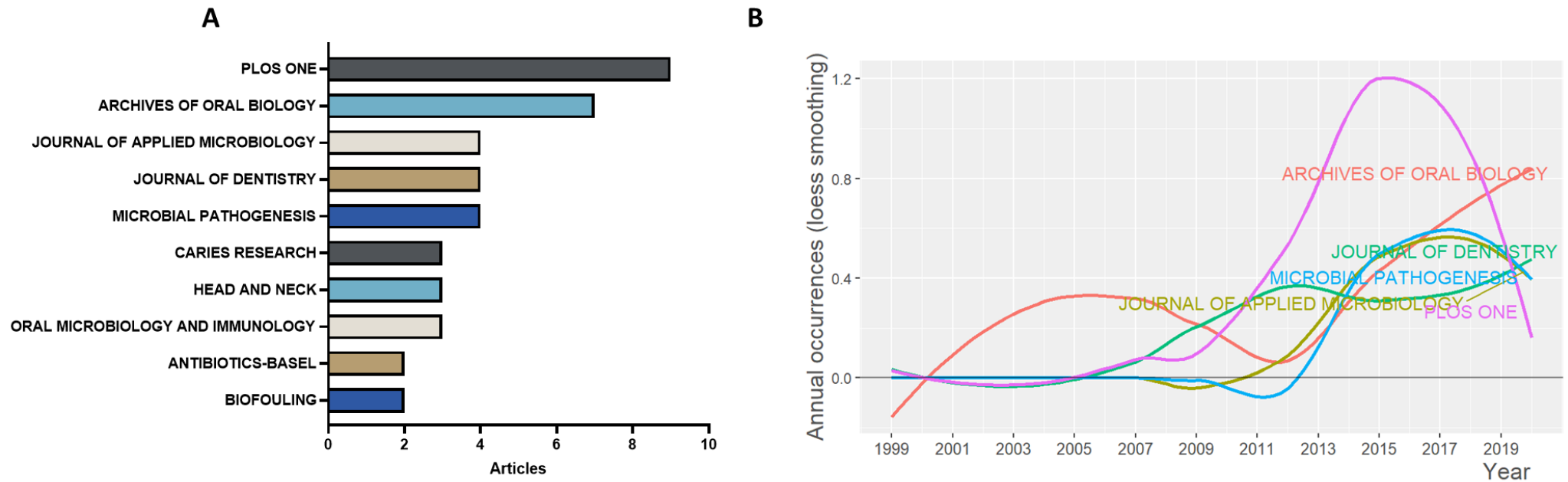


Figure 2.9: Most relevant sources of *Candida* and *Lactobacillus* biofilm research.

Top 10 most relevant sources of scientific literature and number of articles published in *Candida* and *Lactobacillus* biofilm research between 1999-2020 (A).

Dynamics of the top 5 most relevant sources in the field between 1999-2020 (B).

Differences were also apparent when observing relevant authors in the field and their collaborative links compared with the previous search (Figure 2.10). The highest publishing authors in the field were found to be Jorge A. O. C., Junqueira J. C. and Rossoni R. D., each with 7 articles (Figure 2.10A). These authors were also found to have strong collaborative links, appearing together in the red coloured network in Figure 2.10B. Further, other relevant authors were also shown to have close research links, illustrated in the blue network, including Busscher H. J., Van Der Mei H. C, Buijssen K. J. D. A. and Harmsen H. J. M., showing individual research groups.

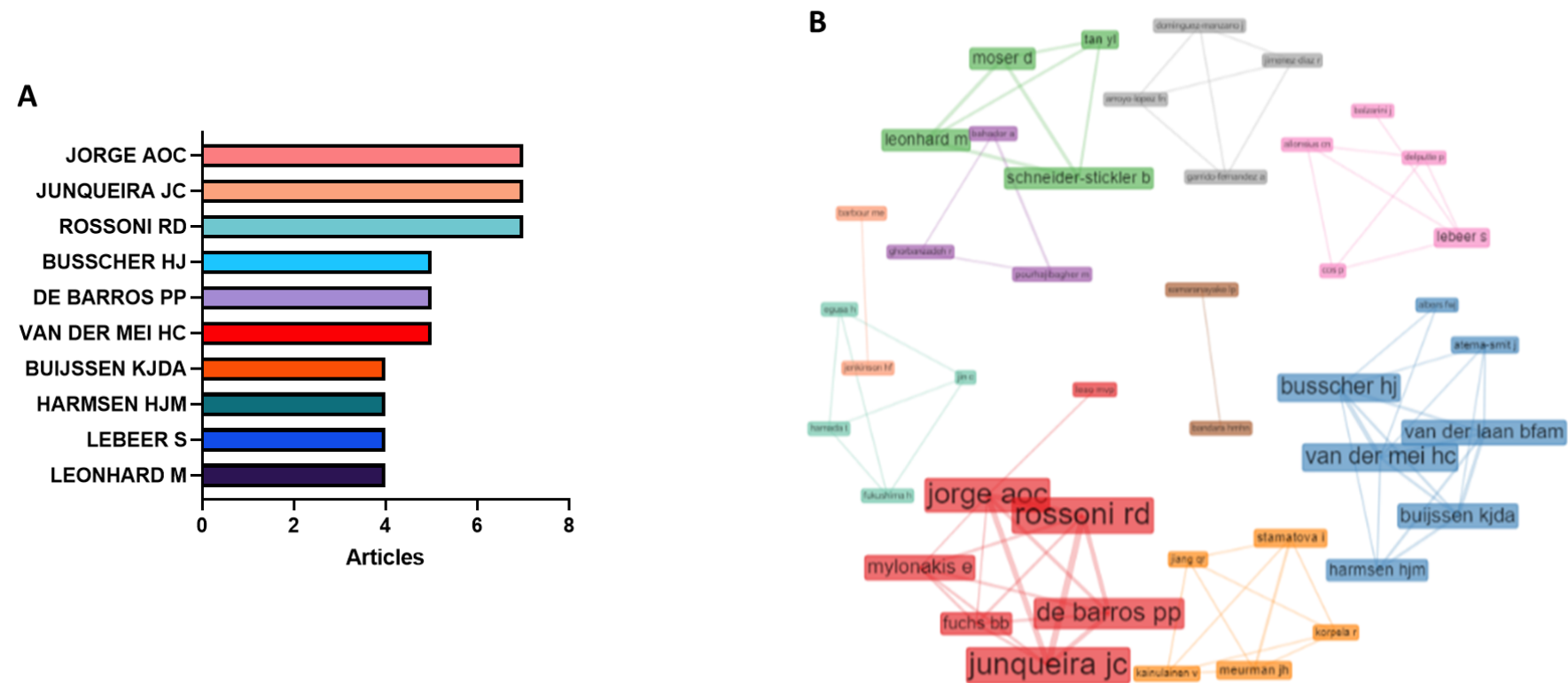


Figure 2.10: Relevant authors and collaborative links in *Candida* and *Lactobacillus* biofilm research.

Relevant authors based on number of published articles in the field between 1999-2020 (A). Network of collaborations between the most published authors (B). Node size is weighted by author contribution; Closed networks of one colour represent closely linked collaborative groups. Isolated nodes were removed.

Production of scientific literature in the field of *Candida* and *Lactobacillus* biofilms was then observed with respect to the corresponding author's home country (Figure 2.11). The majority of research was found to be published in South America, the USA, South-Eastern Asia and the UK, illustrated by a dark blue shading. Areas with a smaller contribution included Australia and areas within Europe. Africa and central Asia were largely found to have no contribution to the field.

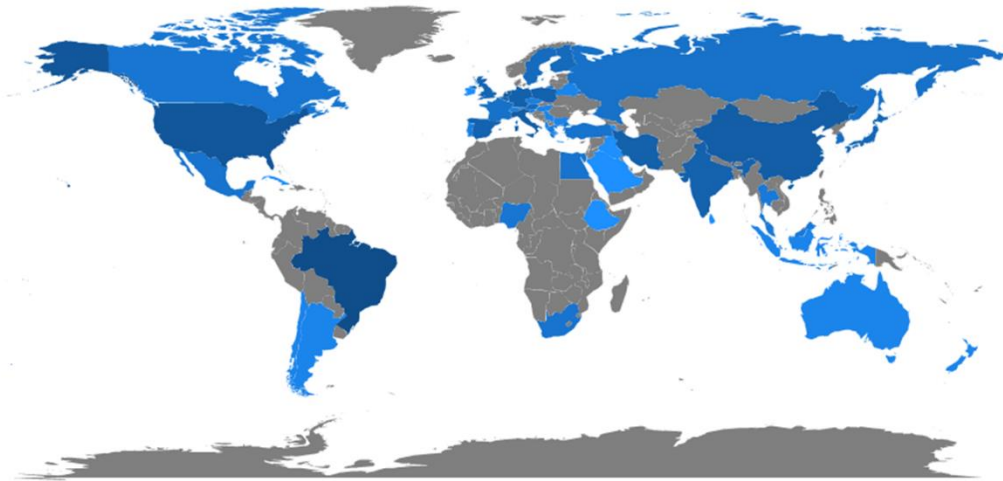


Figure 2.11: Scientific production by country of *Candida* and *Lactobacillus* biofilm research. Scientific contribution by country indicated by dark blue (large contribution), light blue (small contribution) and grey (no contribution) to *Candida* and *Lactobacillus* biofilm research between 1999-2020.

To further investigate the geographical spread of scientific literature, the countries with the highest number of articles and their associated citations are shown in Figure 2.12. Brazil was found to have the highest contribution to the field with a total of 26 articles (Figure 2.12A). Brazil was also found to have the highest level of collaboration with other countries with 7 MCPs. As expected, Brazil was found to have the highest number of total citations, however, with only 23 citations, it had one of the lowest numbers of citations per article (Figure 2.12B). Other relevant countries such as the UK, Germany and China were seen to often publish in collaboration with other countries. Conversely, Italy was found to publish exclusively as SCPs. Despite not being within the top 10 most published countries, the Netherlands was the second most cited country with a substantial 59 average citations per article. Other highly cited countries included the UK, Australia and New Zealand with a combined average 53 citations per article.

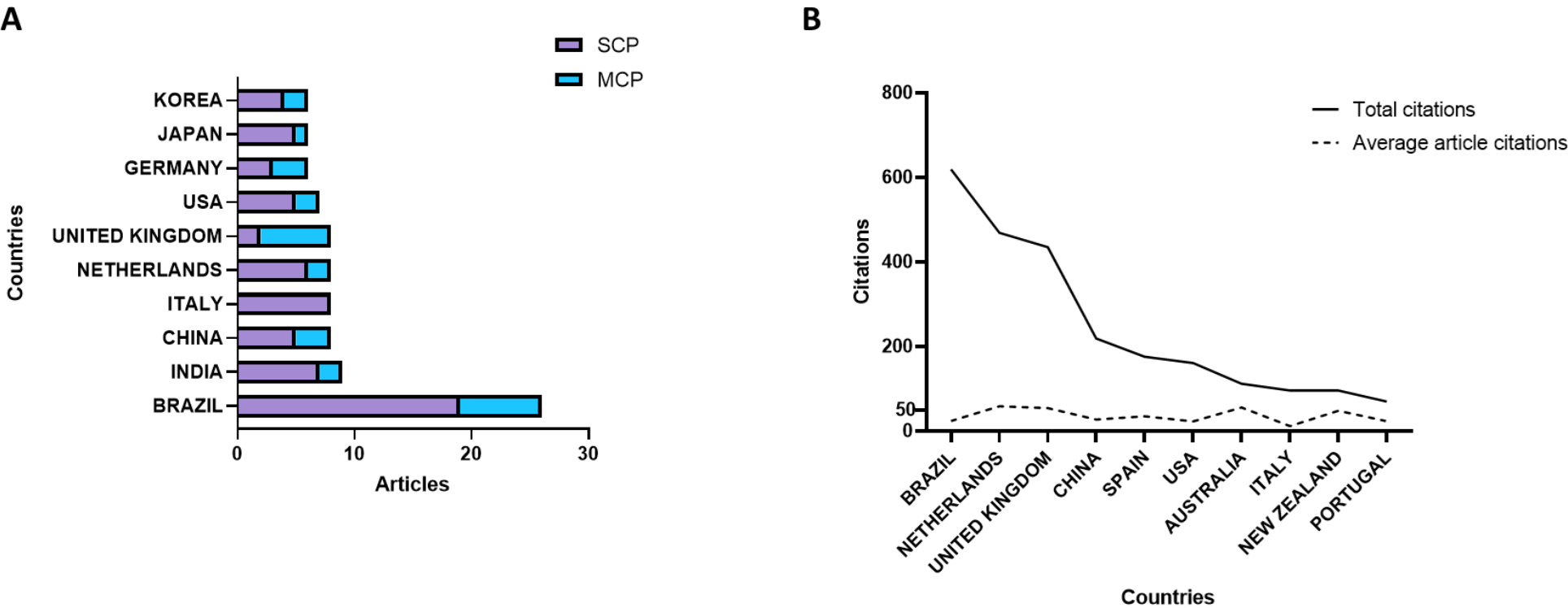


Figure 2.12: Relevance of scientific contribution by country.
Number of articles published by the corresponding author’s country (A). Split bars represent single country publications (SCP) and multiple country publications (MCP). Total and average number of citations from the highest published countries between 1999-2020 (B).

Analysis of frequently used author's keywords and their correlations in *Candida* and *Lactobacillus* biofilm research allowed for an insight into where interest within the field lies (Figure 2.13). Themes including “vulvovaginal candidiasis”, “biofilms” and “bacteriotherapy” were identified as recurring themes in the field (Figure 2.13A). The majority of these themes were found to be associated with keywords related to clinical infection research such as “lactobacilli”, “probiotics” and “antimicrobials” illustrated by the red network in Figure 2.13B. Other themes identified by the thematic map were classed as ‘basic’ themes and include “*Candida albicans*”, “lactobacilli”, “*Streptococcus mutans*” and “biofilm”. These were found to be closely linked themes in the literature, illustrated by significant overlap of ellipses and interlink of keyword networks. Keywords associated with oral microbiology research are shown in purple and include “dental plaque” and “gene expression”. The green network illustrates keywords associated with a range of research including “anti-biofilm activity”, “antimicrobial activity” and “essential oil”.

Finally, a third search was carried out to determine levels of interest in the field of vaginitis in *Candida* and *Lactobacillus* biofilm research (Table 2.1). This search only retrieved 8 research items from the Web of Science Core Collection database, 5 of which were review papers. For this reason, it was not possible to carry out bibliometric analysis using these search terms.

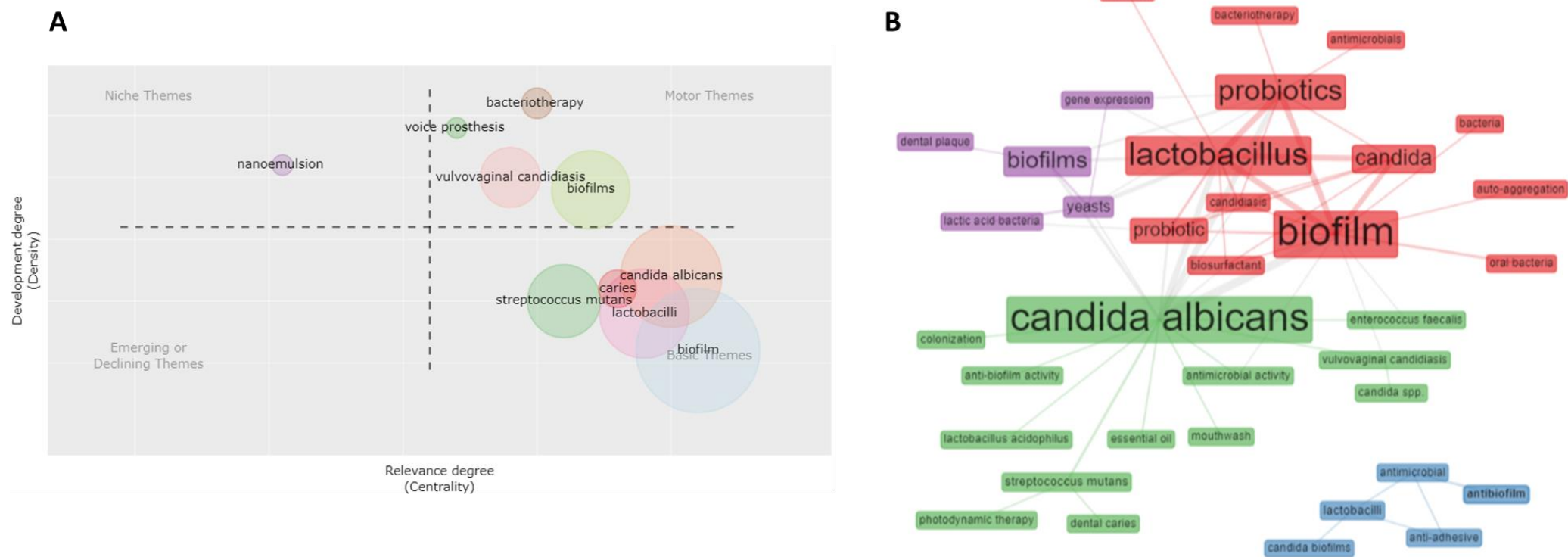


Figure 2.13: Most frequent keywords and their correlations in *Candida* and *Lactobacillus* biofilm research.

Thematic map illustrating the relevance of common keywords in *Candida* and *Lactobacillus* biofilm research (A). Co-occurrence network of the most common author's keywords used in the field (B). Node clustering and size is weighted by co-occurrence and frequency of keywords, respectively. Isolated nodes were removed.

2.4 Discussion

It is now possible for researchers with little knowledge of programming language software to quantify and analyse entire fields of scientific literature through the application of bibliometric analysis using the open-source web application, Biblioshiny®. This ability to gain a wide insight in to developing or declining interest and themes within the field of research greatly expands a researcher's ability to review the area. The analysis in this chapter aimed to identify how research of interactions between *Candida* and *Lactobacillus* has developed over the last three decades, as well as to assess which areas of this research biofilms are most appreciated. In addition, it aimed to investigate these themes with respect to vaginitis.

Assessment of the overall scientific production of literature pertaining to *Candida* and *Lactobacillus* research between 1990-2020 showed a steady increase in interest over time. In-fact, of the 1,860 research items retrieved by the search, 1,142 of these were published in the most recent decade. This is likely a reflection of impactful work published at this time showing *in vitro* and *in vivo* anti-*Candida* activity of both oral and vaginal *Lactobacillus* strains related partly to hydrogen-peroxide production (Strus et al., 2005, Elahi et al., 2005). Further, it is at the beginning of this decade that key publishers in the field including Rossoni R. D. and Jorge A. O. C. began investigating the probiotic potential of *Lactobacillus* species against *C. albicans* in oral candidiasis and their immunological effects in *Galleria mellonella* models. Similarly, the average number of citations per article per year has also increased over this period. The high numbers of total citations on earlier papers published throughout the 1990s is likely due to the later increase in annual publications in the 2000s citing these papers. There are consistently high numbers of citations per article throughout the 2000s, indicative of a sharp increase of interest in the field. Given the steady rise of interest in this area over time, it is likely that numbers of annual publications will continue to increase as understanding of anti-*Candida* capabilities of *Lactobacillus* species grows.

Analysis of relevant sources within the field revealed high numbers of publications in journals associated with food microbiology. This is likely a result of many highly cited publications assessing antagonism between *Candida* and *Lactobacillus* with respect to food spoilage and the fermentation of sourdough (Venturi et al., 2012, Yang and Chang, 2010). However, it is also important to consider the possibility of ease of publication within certain fields as a paper detailing treatment of oral candidiasis with *Lactobacillus* probiotics in a murine model is published in a journal associated with food spoilage (Wagner et al., 2000). Source dynamics over the period from 1990-2020 suggest that the number of publications pertaining to *Candida* and *Lactobacillus* research in the most relevant journals are declining, with an increase in publications in journals such as Food Science and Technology and PLOS One. This data suggests that this field of research is still relevant in both the food industry and in the field of science and medicine and that these journals will likely become the most relevant in years to come.

The most relevant authors in the field were found to have similar numbers of published articles with no one author responsible for the majority of publications. However, this analysis did identify one key group of researchers with close collaborative links, Jorge A. O. C., Junqueira J. C. and Rossini R. D., who have collectively published the most in the field. This group is likely responsible for many publications from Brazil, illustrated by the dark blue coloration on the map in Figure 2.5. Another group of collaborative researchers was identified including Bellen G., Donders G.G.G. and Vitali B. This is an intercountry collaborative group responsible for the majority of publications from Italy and Belgium. Further investigation of each country's contribution revealed China, the USA and Italy as the most highly published countries worldwide. However, despite having the highest volume of publications, China had the lowest total number of citations, suggesting a potential overload of less relevant literature. Although Belgium was not in the top 10 most published countries, it had the highest numbers of citations per article, likely a reflection of the close collaborative links with Italy.

The final analysis of the first bibliometric search aimed to gain insight into common keywords used in *Candida* and *Lactobacillus* research. The most widely used theme identified was “probiotics”; Although this may indicate the treatment of candidiasis

at mucosal sites, common keywords associated with this theme revealed it was primarily associated with research of *Candida* and *Lactobacillus* in the fermentation industry. Overlap of the themes “*Candida albicans*” and “lactic-acid bacteria” was found to be primarily associated with the treatment of oral candidiasis and dental caries, and in-part, sourdough fermentation. Overall, this search revealed a growing interest in *Candida* and *Lactobacillus* research between 1990-2020 with focus on the treatment of oral candidiasis and the prevention of food spoilage during fermentation. The most impactful publications during this time were from two research groups from Brazil and a collaborative group from Belgium and Italy. This data suggests that despite the proven probiotic effects of *Lactobacillus* against *Candida* at mucosal sites, research pertaining to the treatment of vaginal *Candida* infections remained largely understudied during this time.

The next search was carried out to identify which areas of scientific literature involving *Candida* and *Lactobacillus* appreciate the impact of biofilms on clinical treatment and potential microbial interactions. No research items were obtained in this search prior to 1999, as such, subsequent analysis was carried out on articles published between 1999-2020. Very few articles were retrieved throughout the 2000s, with a notable spike in publications in 2015 and 2016. All articles published in this area received high numbers of citations with an average of 25 citations per year. Like the last search, this is likely due to the recent rise in interest in the field resulting in increased citations on earlier articles. The rise in interest from 2015 is in part a reflection of impactful work by Matsubara et al. showing the first insights into the mechanisms of early *Candida* biofilm inhibition by probiotic *L. rhamnosus* (Matsubara et al., 2016b).

The majority of publications in this area were found to be published in journals which publish primarily in oral health and microbiology (Figure 2.9). Similarly to the previous search, PLOS One was found to be the most popular source for research in this field. However, no sources associated with fermentation or food spoilage were identified in this search, suggesting biofilms are not of concern in such areas. Source dynamics revealed a sharp decrease in publications to PLOS One and an increase in those to the Journal of Dentistry and Archives of Oral Biology, however,

given the small number of publications per year, it is difficult to draw definitive conclusions from these data.

The most published authors in the field were similar to the previous search, however, many more collaborative groups were identified. The most published group was Jorge A. O. C.'s research group, again responsible for the high contribution to the field and total citations from Brazil. Another relevant collaborative group was identified from the Netherlands comprising Busscher H. J., Van Der Mei H. C., Buijsen K. J. D. A. and Harmsen H. J. M., who primarily investigate polymicrobial biofilm formation of *Candida* and *Lactobacillus* on voice prosthesis (Buijsen et al., 2007, van der Mei et al., 2014). Despite only 8 publications in the field from the Netherlands over this time, these papers were clearly impactful with 469 total citations. Additionally, an Austrian group who investigate the inhibitory properties of *Lactobacillus* supernatants against *Candida* biofilms in oral candidiasis were identified in this search (Tan et al., 2018). Despite having few publications, the UK was also found to be highly cited in the field of *Candida* and *Lactobacillus* biofilm research.

Finally, analysis of the frequency and co-occurrence of author's keywords in the field revealed various themes. It is clear from the number of widely used and re-occurring themes that this search encompasses many different areas of research. Many of the re-occurring themes including "lactobacilli" "caries" and "*Streptococcus mutans*" shared significant overlap and were associated with keywords such as "mouthwash", "probiotic" and "candidiasis". This data shows the majority of *Candida* and *Lactobacillus* biofilm research focusses on the treatment of oral candidiasis. However, one re-occurring theme was identified as "vulvovaginal candidiasis" and was associated with the keywords "*Candida* spp.", "*Candida albicans*" and "*Enterococcus faecalis*". This shows a clear interest in the role of *Candida* and *Lactobacillus* biofilms in vaginal *Candida* infections, however as a small subsection of the entire field.

Very few articles were retrieved in the third and final search, limiting the amount of analysis performed on this search. This is evident of the lack of appreciation of the impact of *Candida* and *Lactobacillus* biofilms in vaginal infections such as

vulvovaginal candidiasis. There is a clear gap in the literature in the understanding of consequences of interkingdom interactions between *Candida* and *Lactobacillus* in vaginitis. Further research could greatly improve current knowledge of the pathogenesis of *Candida* at mucosal sites, predominantly in the understudied field of vaginal candidiasis. To this end, clinical studies assessing microbial populations in clinical samples of health and RVVC to elucidate potential biofilm formation and microbial interactions associated with disease are required.

Chapter findings

- The relatively novel open-source Biblioshiny® tool provides researchers with a crucial method of handling an expanding pool of scientific literature in a meaningful way, without the need to be proficient in programming language software.
- Interest in literature throughout the last three decades pertaining to *Candida* and *Lactobacillus* research has increased significantly with the publication of highly impactful articles. This research has a strong focus on the fields of food spoilage, fermentation, and oral microbiology.
- More recently, an increased interest in the role of biofilms in *Candida* and *Lactobacillus* research is evident. This interest is primarily focussed on the role and treatment of *Candida* biofilms with *Lactobacillus* probiotics in oral candidiasis, with a significantly smaller focus on vaginal candidiasis

3 Identifying biological indicators and *Candida* biofilm formation in RVVC

3.1 Introduction

Our current understanding of VVC pathology dictates that VVC is an acute inflammatory condition of the vulva and vaginal mucosa due to overgrowth of typically asymptomatic commensal *Candida* species (Fidel et al., 2004). There are many hypothesised predisposing factors to VVC onset, including the use of antibiotics, uncontrolled diabetes mellitus, sexual activities, and tight-fit clothing (Sobel et al., 1998, Sobel, 1997). However, defined triggers for onset and recurrence of symptoms remain largely unknown. It has previously been hypothesised that the relationship between the presence of *Candida* in the vagina and host susceptibility to infection may be due to the sensitivity of the patient's individual vaginal epithelium (Fidel, 2007). This hypothesis means that the 'threshold' for *Candida* load to become symptomatic is different in each patient, also implying a focus on the requirement for personalised medicine. Despite being defined by an overgrowth of *Candida*, many authors have reported variable results when attempting to correlate *Candida* load with severity of VVC (Hopwood et al., 1988, Marot-Leblond et al., 2009). It has been suggested that yeast counts of $>10^3$ CFU/mL indicate symptomatic infection and counts less than this cut-off indicate commensal carriage (Hopwood et al., 1988). However, there are currently no appropriate clinical studies which have investigated this as a potential symptomatic threshold. Conversely, studies have found no correlation between a higher *Candida* load and symptomatic infection (Marot-Leblond et al., 2009). If it is possible to determine the 'infection threshold' for individual patients, it could be possible to prevent development of RVVC through appropriate personalised antifungal prophylactic maintenance therapies.

Until recent decades, the immunopathology of VVC onset was thought to be attributed to a weakened or defective adaptive immune response in patients, similar to oral candidiasis (Klein et al., 1984, Fong et al., 1992). However, studies using murine models failed to determine a crucial role for cell-mediated immunity in the vagina following *Candida* infection (Fidel et al., 1995, Mendling and Koldovsky, 1996). Additionally, an innovative study using human participants demonstrated that *Candida* infection resulted in an extensive non-protective influx of leukocytes during symptomatic VVC (Fidel et al., 2004). This study also determined that a defective

immune response was concurrent with immunity to VVC, contrary to previous beliefs. Since this discovery, many studies have been conducted in an attempt to identify important immune pathways in VVC pathology. Unlike other human *Candida* infections, VVC occurs in people who are immunocompetent and that do not necessarily have underlying health conditions. It is widely accepted that acute inflammation in VVC is not due to an abnormal adaptive immune response and instead is a result of an aggressive innate response by PMNs (Fidel et al., 2004). The chemoattractant responsible for this non-protective influx of PMNs is unknown and studies predominantly focus on utilising murine models to elucidate potential candidates of PMN influx (Yano et al., 2010, Bruno et al., 2015). To date, no studies have assessed inflammatory biomarkers present in clinical samples of VVC/RVVC to expose potential biomarkers of disease. Proteomic analysis is a powerful tool and has been used successfully to identify biological markers for the detection of diseases such as nasopharyngeal carcinoma, meningitis, and infections of the central nervous system (Bharucha et al., 2019, Cordeiro et al., 2015, Yang et al., 2014). Identification of biomarkers for the detection of VVC/RVVC could greatly improve diagnosis accuracy allowing patients to receive appropriate treatments to prevent failure and recurrence.

Diagnosis of VVC is problematic and can result in inappropriate treatment. It has been estimated that up to 77% of VVC cases are misdiagnosed as other inflammatory disorders based on clinical evidence alone (Schwartz et al., 2006). The presence or positive culture of *Candida* itself is not indicative of disease as the commensal carriage rate in the vagina is ~30% (Achkar and Fries, 2010a). Further, current guidelines fail to consider the *Candida* species responsible for VVC which can result in failed clinical treatment and development of debilitating RVVC, as NAC are often less susceptible to conventional antimycotic therapies (Deorukhkar et al., 2014). Of particular clinical concern are *C. glabrata* and *C. krusei* as they are intrinsically highly tolerant to azoles (Singh et al., 2002, Vermitsky and Edlind, 2004). Antifungal ointments and pessaries used to treat VVC are freely available from over-the-counter pharmacies, resulting in a higher likelihood of inappropriate treatment as well as resulting in impossible to determine rates of infection and recurrence. A single dose of oral or topical azole therapy (typically fluconazole) is sufficient to treat sporadic *C. albicans* VVC in 80-90% of cases (Pappas et al., 2009). Treatment of

VVC caused by a NAC species is referred to as complicated VVC, requiring prolonged suppressive azole therapies, exceeding 6 months in some cases, which are frequently unsuccessful contributing to the 8% of women who develop RVVC (Sobel et al., 2003a). In recent years, authors have reported an epidemiological shift in VVC showing a rise of NAC species (Sherry et al., 2017). The most commonly isolated NAC species in VVC is *C. glabrata* followed by *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. krusei*, *C. kefyr* and *C. guilliermondii* (Deorukhkar et al., 2014). The global prevalence of NAC species in VVC has previously been estimated to be 10-20%, however this is highly geographically dependant (Fidel et al., 1999). Generally, there are very few reports of the prevalence of NAC species within women in the UK. However, our group has previously observed a prevalence of 29% NAC species among women with VVC in the UK (Sherry et al., 2017). The lowest proportions of NAC species reported have been in China, Brazil, Belgium, Italy and Turkey which all reported >75% of VVC episodes caused by *C. albicans* (Shi et al., 2015, Ribeiro et al., 2001, De Vos et al., 2005, Corsello et al., 2003a, Ozcan et al., 2006). Conversely, studies have shown countries such as India, Nigeria, and Ethiopia to have higher rates of NAC species episodes, accounting for up to 40% of total cases (Ahmad and Khan, 2009, Bodenham et al., 2020, Bitew and Abebaw, 2018). This data highlights the importance of consideration of the causative species of *Candida* in individual cases of VVC before decisions for clinical treatment are made.

Despite the ability of *Candida* species to readily form dense biofilms and their impact on clinical treatment (Ramage et al., 2012a), the presence of biofilms on vaginal epithelium in VVC/RVVC is disputed by some authors (Swidsinski et al., 2019, Sobel, 2015b). Although they have been shown to form both *in vitro* and *in vivo*, vaginal *Candida* biofilms remain unrecognised when making decisions to treat VVC (Harriott et al., 2010b, Sherry et al., 2014, Paiva et al., 2012). Unfortunately, there are no vaginal biopsy studies for VVC on a scale similar to those conducted for BV research, and as such the hypothesis of vaginal biofilms in VVC remains unclear. Although some studies have identified biofilm-related gene expression in clinical isolates of VVC (Khosravi Rad et al., 2016, Gao et al., 2016), none have aimed to find evidence of expression of these genes in clinical samples of VVC/RVVC.

Hypotheses and aims

Despite exceptionally high case rates of up to 75% of all women of child-bearing age, the triggers for onset of VVC/RVVC remain largely unknown. Additionally, possible causes of failed clinical treatment are understudied in the field and better understanding could greatly improve treatment prospects for women with recurrent, azole-resistant episodes. Therefore, the main aims of this chapter were to:

- Gain insights in to states of health and RVVC through analysis of demographic data from patients recruited for this study.
- Observe and compare the immunological and microbiological biomarkers of healthy patients and those with RVVC.
- Evaluate the presence of vaginal biofilms and their impact on RVVC treatment.
- Assess alternative potential sources of failed clinical treatment reported in RVVC.

It is hypothesised, based on previous research, that an increased fungal load and elevated levels of inflammatory biomarkers in samples from diseased women will be indicative of RVVC. Additionally, it is theorised that the ability of *Candida* clinical isolates to form biofilms with increased antifungal tolerance and express key biofilm-related genes, has the potential to cause failed treatment in clinical settings.

Work from this chapter has been presented at the following workshops/conferences:

University of Glasgow postgraduate prize seminar, May 2019, Glasgow, UK

Eurobiofilms Conference, September 2019, Glasgow, UK

Federation of Infection Societies Conference, November 2019, Edinburgh, UK

Work from this chapter has been published in mSystems:

McKloud E, Delaney C, Sherry L, Kean R, Williams S, Metcalfe R, Thomas R, Richardson R, Gerasimidis K, Nile CJ, Williams C, Ramage G. Recurrent Vulvovaginal Candidiasis: A Dynamic Interkingdom Biofilm Disease of *Candida* and *Lactobacillus*. mSystems. 2021 Aug 10:e0062221. doi: 10.1128/mSystems.00622-21.

3.2 Materials and methods

3.2.1 Clinical sample processing and analysis

3.2.2 Patient recruitment

One hundred women aged 18 and over attending Glasgow Sandyford Sexual Health Clinic between February 2017 and June 2018 were enrolled in the study. Patients presenting with symptomatic RVVC were recruited (n=40) as well as healthy women attending the clinic for contraceptive intrauterine device (IUD) implantation as a control cohort (n=60). This removed bias of the study to solely recruit women with symptomatic disease. This study was granted ethical approval by the Sheffield Research Ethics Committee (16/YH/0310). Dr Rebecca Metcalfe (Specialist Registrar), or another doctor trained in this study was responsible for patient recruitment and obtaining informed consent from prospective participants after they had read the patient information sheet. If the patient chose to participate, a questionnaire was completed and given to the clinician in charge of the study, Dr Rebecca Metcalfe. Dr Rebecca Metcalfe or a qualified research nurse was responsible for sample collection during routine examination. Patients were excluded from the study if they had active bacterial vaginosis, were pregnant, immunosuppressed, menstruating, menopausal or had taken antibiotics/antifungals within 7 days prior to sampling.

3.2.3 Collection of clinical samples

During routine examination, a sterile speculum was used to obtain two high vaginal swabs (HVS) from each patient. One swab formed part of routine care which was sent for organism identification at the NHS Greater Glasgow and Clyde (GGC) labs, the other was used in this study. Additionally, a cervico-vaginal lavage (CVL) was collected using 5 mL of sterile water, allowing the fluid to pool prior to removal. Swab samples were submerged in 500 µL of PCR grade water, contained within a universal collection tube. Samples were then vortexed for 30 s before being transferred to cryovials and stored at -80°C. Lavage samples were transferred to cryovials and stored at -80°C. Supernatants collected from lavage samples were used to measure vaginal pH.

3.2.4 Collection of patient data

Potential participants attending the clinic were either referred by their GP or self-referred. Once in the clinic, they were provided information on the study and if informed consent was obtained, they were provided with a questionnaire (See appendices i-v). The patient questionnaire collected data on patient's RVVC history, including number of episodes within the previous 12 months, treatment regimens, and any contraception currently used by the patient. Patients were allocated a unique study code using a numbering system with a letter to identify disease status (SVS001A for the first healthy patient, SVS001B for the first RVVC patient, etc.) which was used to identify each sample during subsequent analysis. A graphical representation of sample collection and processing can be found in Figure 3.1.

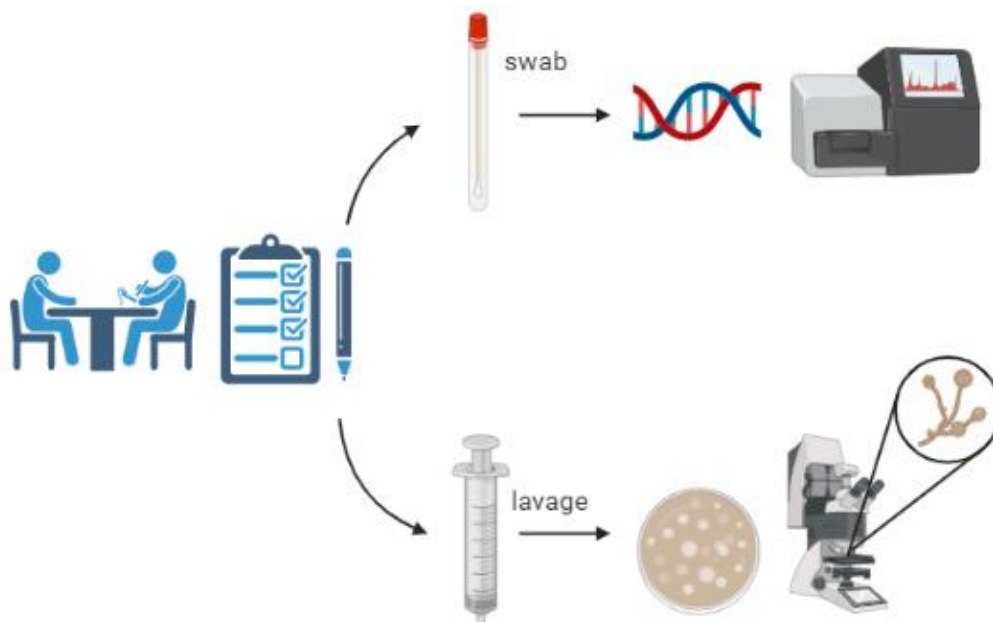


Figure 3.1: Illustrative representation of clinical study sample collection and processing. After distribution of information sheets and collection of informed consent, patient questionnaires were completed. Both high-vaginal swab and cervico-vaginal lavage samples were collected. Swab samples were used to extract DNA for microbial profiling. Lavage samples were used for species ID and isolation and visualisation of *Candida*. Created with Biorender.com.

3.2.5 Quantification of microbial load by real-time PCR

Candida and bacterial burdens were determined by extracting DNA from swab samples and analysing using qPCR. Samples were transferred to Eppendorf tubes and centrifuged, the resulting pellet was then enzyme-treated with lysozyme (10

mg/mL in 10 mM Tris-HCL pH 8) for 1 h at 37°C and proteinase K (20 mg/mL in 5.5% SDS) for 1 h at 55°C. DNA was extracted using the QIAmp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK) and qPCR used to quantify fungal and bacterial load in each sample. Primers specific to the conserved *Candida* ITS rRNA gene were used to determine *Candida* load. For bacterial load, primers and a Taqman probe specifically targeting the prokaryotic 16S rRNA gene were used. Primer sequences are presented in Table 3.1.

Table 3.1: qPCR primer sequences for microbial quantification

Gene name	Primer sequence (5' – 3')
<i>ITS3</i>	F – TCGCATCGATGAAGAACGCAGC
<i>ITS4</i>	R – TCTTTTCCTCCGCTTATTGATATGC
<i>16S</i>	F – TCCTACGGGAGGCAGCAGT
	R - GGACTACCAGGGTATCTAATCCTGTT
	Probe – CGTATTACCGCGGCTGCTGGCAC

Total qPCR reaction volume was 20 µL, with 1 µL of extracted DNA, 500 nM of forward/reverse primers and UV-treated RNase free H₂O. For 16S, 250 nM of probe and 2X Taqman™ Universal PCR Master Mix (ThermoScientific, Loughborough, UK) was used, 2X Fast SYBR™ Green PCR Master Mix (ThermoScientific, Loughborough, UK) was used for ITS primers. qPCR was performed using an Applied Biosystems™ StepOne™ Real-Time PCR System (Life Technologies, Paisley, UK) with the following thermal conditions: an activation step of 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Standard curves constructed from serially diluted DNA of *C. albicans* SC5314 (Appendix 5) and *Escherichia coli* K12 (Appendix 6) were used extrapolate *Candida* and bacterial colony forming equivalents (CFE/mL), respectively, as described previously (O'Donnell et al., 2015).

3.2.6 Detection of inflammatory biomarkers in CVL with Olink Proteomics

CVL supernatants were recovered by centrifugation for proteomic analysis. Initially, 10 healthy and 10 samples from RVVC patients were selected and analysed using the commercially available Olink Proseek™ Multiplex Inflammation I (v.3012) panel (Olink Proteomics, Uppsala, Sweden) (Assarsson et al., 2014, Moen et al., 2016).

This panel screens for the presence of 92 preselected proteins related to inflammation (Table 3.2). A proximity extension assay (PEA) was performed, allowing for high throughput multiplexing to measure levels of 92 biomarkers simultaneously.

Table 3.2: List of inflammatory biomarkers detected by Olink proteomic panel.

Protein	Gene	UniProt ID
Interleukin-8	IL8	P10145
Vascular endothelial growth factor A	VEGFA	P15692
T-cell surface glycoprotein CD8 alpha chain	CD8A	P01732
C-C motif chemokine 7	MCP-3	P80098
Glial cell line-derived neurotrophic factor	GDNF	P39905
CUB domain-containing protein 1	CDCP1	Q9H5V8
Natural killer cell receptor 2B4	CD244	Q9BZW8
Interleukin-7	IL7	P13232
Tumor necrosis factor receptor superfamily member 11B	TNFRSF11E	O00300
Transforming growth factor beta-1 proprotein	TGFB1	P01137
Urokinase-type plasminogen activator	PLAU	P00749
Interleukin-6	IL6	P05231
Interleukin-17C	IL-17C	Q9P0M4
C-C motif chemokine 2	CCL2	P13500
Interleukin-17A	IL-17A	Q16552
C-X-C motif chemokine 11	CXCL11	O14625
Axin-1	AXIN1	O15169
Tumor necrosis factor ligand superfamily member 10	TNFSF10	P50591
Interleukin-20 receptor subunit alpha	IL20RA	Q9UHF4
C-X-C motif chemokine 9	CXCL9	Q07325
Cystatin-D	CST5	P28325
Interleukin-2 receptor subunit beta	IL-2RB	P14784
Interleukin-1 alpha	IL-1 alpha	P01583
Oncostatin-M	OSM	P13725
Interleukin-2	IL2	P60568
Growth-regulated alpha protein	CXCL1	P09341
Thymic stromal lymphopoietin	TSLP	Q969D9
C-C motif chemokine 4	CCL4	P13236
T cell surface glycoprotein CD6 isoform e	CD6	Q8WWJ7
Kit ligand	KITLG	P21583
Interleukin-18	IL18	Q14116
Signaling lymphocytic activation molecule	SLAMF1	Q13291
Protransforming growth factor alpha	TGFA	P01135
C-C motif chemokine 13	CCL13	Q99616
Eotaxin	CCL11	P51671
Tumor necrosis factor ligand superfamily member 14	TNFSF14	O43557
Fibroblast growth factor 23	FGF-23	Q9GZV9
Interleukin-10 receptor subunit alpha	IL-10RA	Q13651
Fibroblast growth factor 5	FGF-5	Q8NF90
Interstitial collagenase	MMP-1	P03956
Leukemia inhibitory factor receptor	LIF-R	P42702
Fibroblast growth factor 21	FGF-21	Q9NSA1

C-C motif chemokine 19	CCL19	Q99731
Interleukin-15 receptor subunit alpha	IL-15RA	Q13261
Interleukin-10 receptor subunit beta	IL-10RB	Q08334
Interleukin-22 receptor subunit alpha-1	IL-22 RA1	Q8N6P7
Interleukin-18 receptor 1	IL-18R1	Q13478
Programmed cell death 1 ligand 1	CD274	Q9NZQ7
Beta-nerve growth factor	NGF	P01138
C-X-C motif chemokine 5	CXCL5	P42830
Tumor necrosis factor ligand superfamily member 11	TNFSF11	O14788
Hepatocyte growth factor	HGF	P14210
Interleukin-12 subunit beta	IL-12B	P29460
Interleukin-24	IL-24	Q13007
Interleukin-13	IL13	P35225
Artemin	ARTN	Q5T4W7
Stromelysin-2	MMP10	P09238
Interleukin-10	IL10	P22301
Tumor necrosis factor	TNF	P01375
C-C motif chemokine 23	CCL23	P55773
T-cell surface glycoprotein CD5	CD5	P06127
C-C motif chemokine 3	CCL3	P10147
Fms-related tyrosine kinase 3 ligand	Flt3L	P49771
C-X-C motif chemokine 6	CXCL6	P80162
C-X-C motif chemokine 10	CXCL10	P02778
Eukaryotic translation initiation factor 4E-binding protein 1	EIF4EBP1	Q13541
Interleukin-20	IL20	Q9NYY1
NAD-dependent protein deacetylase sirtuin-2	SIRT2	Q8IXJ6
C-C motif chemokine 28	CCL28	Q9NRJ3
Delta and Notch-like epidermal growth factor-related receptor	DNER	Q8NFT8
Protein S100-A12	S100A12	P80511
Tumor necrosis factor receptor superfamily member 5	CD40	P25942
Interleukin-33	IL33	O95760
Interferon gamma	IFNG	P01579
Fibroblast growth factor 19	FGF-19	O95750
Interleukin-4	IL4	P05112
Leukemia inhibitory factor	LIF	P15018
Neurturin	NRTN	Q99748
C-C motif chemokine 8	CCL8	P80075
Caspase-8	CASP-8	Q14790
C-C motif chemokine 25	CCL25	O15444
Fractalkine	CX3CL1	P78423
Tumor necrosis factor receptor superfamily member 9	TNFRSF9	Q07011
Neurotrophin-3	NTF3	P20783
Tumor necrosis factor ligand superfamily member 12	TNFSF12	O43508
C-C motif chemokine 20	CCL20	P78556
Sulfotransferase 1A1	SULT1A1	P50225
STAM-binding protein	STAMPB	O95630
Interleukin-5	IL5	P05113
Adenosine deaminase	ADA	P00813
Lymphotoxin-alpha	LTA	P01374
Macrophage colony-stimulating factor 1	CSF-1	P09603

Briefly, two oligonucleotide-labelled antibodies (or “probes”) bind to the target protein in the sample. The proximity of the probes to each other allows a new PCR target sequence to form using a proximity-dependant DNA polymerase. This sequence is then detected and quantified using qPCR to assess biomarker levels. A graphical overview of this process is presented in Figure 3.2.

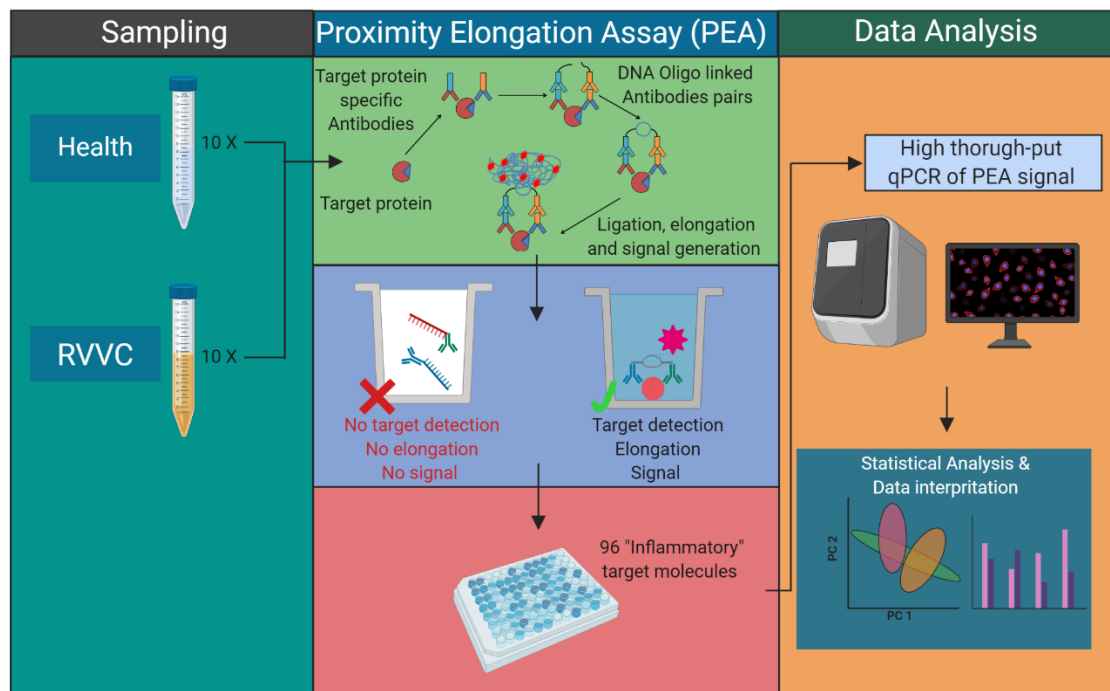


Figure 3.2: Graphical illustration of Olink biomarker detection and analysis pipeline.
Created with Biorender.com.

3.2.7 Detection of inflammatory biomarkers by ELISA

Three of the inflammatory chemokines, CXCL9, CXCL10 and IL-8, detected at significantly higher levels in RVVC compared with health were analysed in all CVL samples by Enzyme-Linked Immunosorbent Assay (ELISA) (Invitrogen, Paisley, UK). For detection of levels of IL-8, 96-well plates (Costar 9018, Corning, USA) were coated with anti-human IL-8 antibody (1:250 dilution) overnight at 4°C. Following washing with 250 µL of 1× PBS, 0.05% Tween™ 20 3-5 times and blocking non-specific sites for 1 h, 100 µL of 1:2 diluted CVL supernatant samples and IL-8 standards (250 – 3.9 pg/mL) were added and incubated at room temperature for 2 h. Plates were washed before addition of biotin-conjugated anti-human IL-8 antibody (1:250 dilution) for 1 h, followed by another wash step and addition of avidin bound to the enzyme, Horse Radish Peroxidase (HRP) (1:250 dilution) for 30 min. Plates

were washed a final time and 1× tetramethylbenzidine (TMB) substrate was added, colour was allowed to develop for around 15 min before 50 µL of 1 M HCl was added to stop the reaction. Absorbance was then measured using a spectrophotometer at 450 nm and 570 nm.

For detection of CXCL9 and CXCL10, ELISA kits pre-coated with anti-human CXCL9/CXCL10 antibody were used. Briefly, CVL samples (1:2 dilution) and standards were added to 96-well plates (Costar 9018, Corning, USA) and incubated at room temperature for 2.5 - 3 h. Biotinylated antibody targeting CXCL9/CXCL10 was then added, and plates incubated for 1 h before addition of streptavidin-HRP for 30 min. After a final wash step, TMB (CXCL9) or stabilised chromogen (CXCL10) was incubated for around 30 min before 1 M HCL was added to stop the reaction. Absorbances were read spectrophotometrically at 450 nm and 550 nm. Standard curves were constructed using the absorbance readings for the standards and biomarker levels in CVL samples were calculated from the curve and multiplied by the appropriate dilution factor. Each sample was assessed in triplicate and appropriate negative controls were included throughout.

3.2.8 Clinical isolate in vitro biofilm formation

3.2.9 Visualisation of *C. albicans* filamentation in CVL

For visualisation of *Candida* aggregates, 30 µL of CVL from a patient with RVVC was stained with calcofluor white (CFW) (Invitrogen, Paisley, UK) to a final concentration of 0.06 µg/mL. CFW stains cellulose and chitin in the fungal cell wall allowing for fluorescent visualisation of both yeast cells and hyphae. Samples were incubated for 1 h at 37°C before being imaged using the EVOS live cell imaging system at an excitation/emission wavelength of 357/447nm (ThermoScientific, Loughborough, UK).

3.2.10 Identification of *Candida* species from cervico-vaginal lavage

Samples were screened for presence and identification of *Candida* species using Colorex *Candida* chromogenic agar (E&O Laboratories Ltd, Bonnybridge, UK) and Matrix Assisted Laser Desorption/Ionization – Time Of Flight (MALDI-TOF) mass spectrometry. For culture identification, 20 µL of CVL sample was spread across the

surface of a chromogenic agar plate before 48 h incubation at 30°C. The colour of the colonies cultured was used to determine *Candida* species, colony numbers were used to calculate the number of colony forming units per millilitre (CFU/mL). Purity plates were made for each *Candida* isolate by sub-culturing 3 distinct colonies from the chromogenic agar plate to fresh Sabouraud dextrose agar (SAB [Merck, Gillingham, UK]) and incubating for 48 h at 30°C. Clinical isolates were then stored on beads in glycerol in Microbank® vials (Pro-Lab Diagnostics, Cheshire, UK) at -80°C.

As a more accurate method of identification, MALDI-TOF mass spectrometry was used to confirm the identify of all cultured species. MALDI-TOF analysis was performed at Glasgow Royal Infirmary laboratories under the supervision of Dr Mary Ann Ritchie. *Candida* isolates were cultured from beads to SAB agar plates and incubated for 48 h at 30°C to achieve single colony growth. Isolate identities were confirmed by MALDI-TOF analysis using a Bruker Microflex, comparing recorded spectra to the Bruker database. Briefly, MALDI-TOF analysis for yeasts involves pre-treating the cells with 70% formic acid, coating the sample for detection with a matrix and allowing it to crystalise. The sample entrapped within the matrix is then ionised using a laser, generating singly protonated ions from the sample. These ions are then fired at a fixed point where and can be measured based on the time taken for the ion to travel to the end of the flight tube and pass the detector (Singhal et al., 2015).

3.2.11 Screening of RVVC clinical isolates for biofilm formation

Candida clinical isolates were assessed for biofilm forming capabilities by crystal violet and [2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide] (XTT) metabolic activity assays (Kuhn et al., 2003, Feoktistova et al., 2016). *Candida* isolates (n=33) were cultured from lavage samples on SAB agar for 48 h at 30°C. For biofilm formation, overnight cultures were grown in yeast extract peptone dextrose (YPD) at 30°C with shaking at 180rpm. Cultures were washed twice with PBS and standardised in RPMI 1640 medium (Merck, Gillingham, UK) to a final cell density of 1×10^6 CFU/mL. Eight biofilms of each isolate were grown in 96-well, flat bottomed microtitre plates for 24 h at 37°C before washing and metabolic activity was measured by the (XTT) reduction assay (Pierce et al., 2008).

Briefly, 100 µL of XTT with 1 µM menadione was added to each biofilm and incubated at 37°C for 90 min before 75 µL of each supernatant was transferred to a fresh 96-well flat-bottomed microtitre plate and metabolic activity measured spectrophotometrically at 492 nm. Biofilms were then allowed to dry for 24 h at room temperature before biomass was measured by the crystal violet assay (Ramage et al., 2001a). Briefly, wells were flooded with 0.05% crystal violet solution for 20 min at room temperature. The stain was removed, and biofilms washed with water to remove excess stain before 100 µL of 100% ethanol was added to remove bound stain from biofilms. 75 µL of supernatant was then transferred to a fresh 96-well flat-bottomed microtitre plate and biomass measured spectrophotometrically at 570 nm. For each isolate, 8 biofilms were assessed, and negative controls were included throughout.

3.2.12 RVVC clinical isolate antifungal susceptibility testing

For antifungal susceptibility testing of *Candida* clinical isolates, overnight cultures were washed and standardised in RPMI 1640 media to a final cell density of 2×10^4 CFU/mL for planktonic cells and 1×10^6 CFU/mL for sessile cells. For planktonic testing, fluconazole, miconazole and clotrimazole were made to a working concentration of 64 µg/mL in RPMI 1640 media before 200 µL was added to the first column of a 96-well round-bottomed microtitre plates and serially diluted from 64 µg/mL to 0.126 µg/mL in RPMI 1640 medium. Then, 100 µL of *Candida* suspensions were added and treatments were incubated for 24 h at 37°C before minimum inhibitory concentrations (MICs) were visually assessed at 50% and 90% inhibition, respectively. For biofilm susceptibility, 200 µL of *Candida* suspensions were added to 96-well flat-bottomed microtitre plates and incubated at 37°C for 24 h to allow biofilm formation. Biofilms were then washed twice with 200 µL PBS before addition of 200 µL of treatment was added, as described above, and serially diluted in RPMI 1640 from 32 µg/mL to 0.063 µg/mL. Treatments were then incubated at 37°C for a further 24 h. Treatments were then removed, and biofilms washed twice with PBS before sessile MICs determined using the XTT metabolic reduction assay, as described above. Ten technical replicates were performed for each isolate assessed. Positive untreated controls and negative media controls were included throughout.

3.2.13 Biofilm-related transcriptional analysis of cervico-vaginal lavage samples

CVL samples were used to analyse expression levels of key *Candida* biofilm-related genes. Samples were centrifuged, and supernatants recovered before pellets were stored in RNeasy[®] (QIAGEN, Manchester, UK) at -80°C. To extract RNA, pellets were subjected to enzyme treatment using lysozyme and proteinase K before cells were homogenised using lysis buffer and 3 × 30 s cycles in a BeadBug[™] microtube homogeniser (Merck, Gillingham, UK). RNA was then purified using the PureLink[™] RNA Mini kit (ThermoScientific, Loughborough, UK), as per manufacturer's instructions and quality and quantity was assessed using a Nanodrop spectrophotometer (ND-1000, ThermoScientific, Loughborough, UK). High quality RNA was retained if the 260/280 value was between 1.8 - 2.2. cDNA was then synthesised from 160 ng of total RNA using a High-Capacity cDNA Reverse Transcription kit (Life Technologies, Paisley, UK) in a MyCycler PCR machine (Bio-Rad Laboratories, Hertfordshire, UK), following manufacturer's instructions. Cycle conditions were 10 min at 25°C, 2 h at 37°C and 5 min at 85°C, samples were then held at 4°C. For samples which contained <16 ng/μL RNA (4.02 – 15.5 ng/μL) after extraction, 10 μL of undiluted RNA was added to the cDNA reaction. No reverse transcriptase controls were included.

Genes assessed included *ECE1* which encodes candidalysin and has been extensively studied for its crucial involvement in *Candida* pathogenicity in a range of infection models including oral candidiasis (Birsel et al., 1993, Moyes et al., 2016). *HWP1* (Hyphal Wall Protein) is an adhesin involved in hyphal morphogenesis and tissue invasion in *Candida* infections (Sundstrom et al., 2002). *ALS3* (Agglutinin-Like Sequence) is another adhesin and plays an important role in attachment to epithelium and in *Candida* biofilm formation (Liu and Filler, 2011). *SAPs* (secreted aspartyl proteinases) expressed by *Candida* are extracellular hydrolytic enzymes involved in invasion, tissue damage and evasion of host response (Hube, 1996). Primers were selected and are listed in Table 3.3. qPCR reactions were constructed with 160 ng of RNA, 2× Fast SYBR[®] Green PCR Master Mix (ThermoScientific, Loughborough, UK), 10μM forward/reverse primers and UV-treated RNase-free H₂O to carry out qPCR with the following cycle conditions: 2 min at 50°C, 2 min at 95°C and 40 cycles of 10 s at 95°C and 30 s at 60°C. Reactions were carried out in duplicate and no reverse transcription (NRT) and no template controls (NTC) were

included throughout. Gene expression was normalised to the *ITS* housekeeping gene and calculated using the $\Delta\Delta C_t$ method (Winer et al., 1999).

Table 3.3: qPCR primer sequences for gene expression analysis

Gene name	Primer sequence (5' – 3')
<i>HWP1</i>	F - GCTCAACTTATTGCTATCGCTTATTACA R - GACCGTCTACCTGTGGGACAGT
<i>ECE1</i>	F - GCTGGTATCATTGCTGATAT R - TTCGATGGATTGTTGAACAC
<i>ALS3</i>	F - CAACTTGGGTTATTGAAACAAAAACA R - AGAAACAGAAACCCAAGAACAACCT
<i>ACT1</i>	F - AAGAATTGATTTGGCTGGTAGAGA R - TGGCAGAAGATTGAGAAGAAGTTT

3.2.14 Statistical analysis

Figures for analysis of Olink proteomic data were constructed in RStudio (Version 3.12.0). All other figures and analyses were performed in GraphPad Prism (Version 8, La Jolla, California, USA). Statistical significance was measured using unpaired t-tests with Welch's correction to compare the means of two samples and regression or Mann-Whitney if data was not normally distributed. One-way ANOVAs were used to compare the means of multiple samples. Statistical significance was achieved if $P < 0.05$.

3.3 Results

3.3.1 Clinical sample processing and analysis

Swab and lavage samples were collected from 100 patients, 60 of which were healthy individuals and 40 were women suffering from RVVC. Demographic data collected through the patient questionnaire and presence/absence of *Candida* is reported in Table 3.4. Additionally, data related to types of contraception used at the time of sampling were collected and used for correlation analysis.

Table 3.4: Patient demographics of women recruited for the study.

Disease Status		Duration of episode	Time since last treatment	Culturable <i>Candida</i> (%)	Detectable <i>Candida</i> (%)
Participants n=100		(Months) (Mean \pm SD)	(Months) (Mean \pm SD)		
Health	60	N/A	N/A	9 (15)	60 (100)
RVVC*	40	8 \pm 3 (min: 3, max: 12)	1 \pm 0.9 (min: 0.25, max: 4)	24 (60)	38 (95)

*Patients defined as suffering from RVVC had presented at GP clinics with symptoms of VVC four or more times within one year.

Vaginal dysbiosis is often alleged to coincide with an elevated vaginal pH in diseases such as BV and occasionally in RVVC.

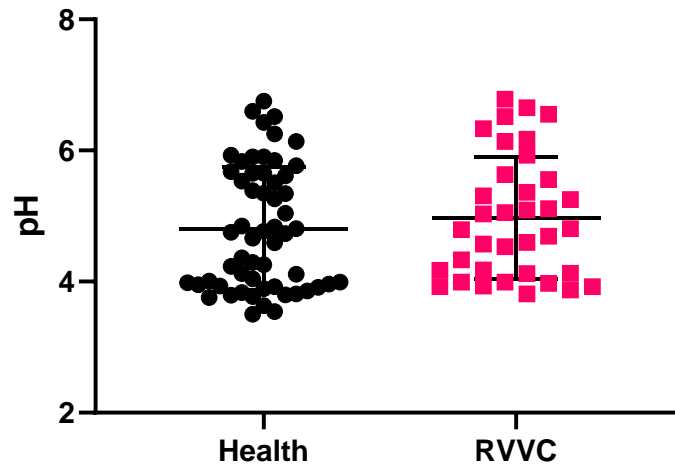


Figure 3.3: The pH of vaginal lavage is unchanged in RVVC. Vaginal lavage supernatants were recovered by centrifugation before the pH was measured (n=93). Data represents mean \pm SD.

Assessment of vaginal lavage supernatant pH levels observed no significant differences between health and RVVC in this study (Figure 3.3). Both cohorts were found to range from a pH of ~3.5 to ~6.7. The mean pH of healthy samples was observed at 4.81 and 4.97 for RVVC samples ($P=0.43$).

3.3.2 Quantification of microbial load

The mechanisms behind the shift in *Candida* from asymptomatic commensal to pathogenic yeast seen in VVC onset remains poorly understood. The events leading to infection are likely multifactorial and here we aimed to assess whether fungal load may influence disease state. To this end, levels of *Candida* present in the swab samples were measured using both the plate-dilution method to assess culturable *Candida* and by real-time qPCR via amplification of the ITS region (Figure 3.4). Additionally, levels of bacteria were also measured molecularly in swab samples to assess potential differences between the two cohorts and to allow for normalisation of bacterial-*Candida* levels (Figure 3.5).

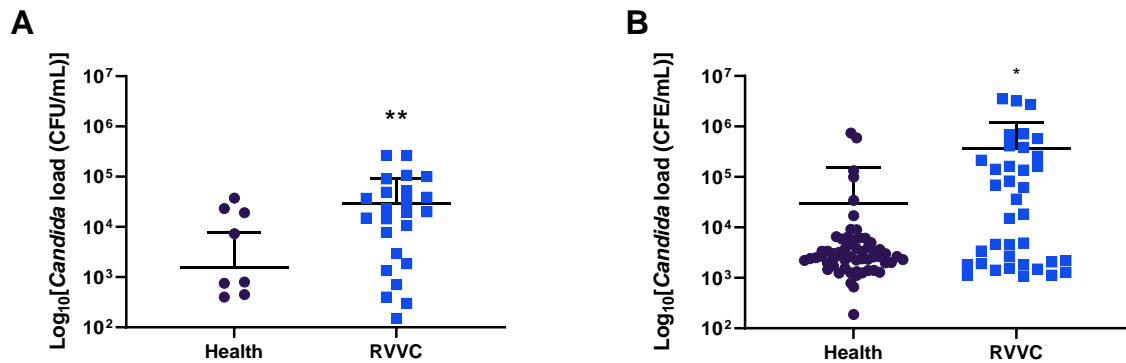


Figure 3.4: Fungal burden is elevated in women with RVVC. To determine fungal load, patient lavage was incubated on *Candida* chromogenic agar plates and CFU/mL evaluated after 48 h (A) For calculation of CFE/mL, the ITS region of *Candida* was amplified using genus-specific primers (B). Data represents the mean \pm SD (*, $P < 0.05$, **, $P < 0.01$); Statistical significance was calculated using unpaired t-tests with Welch's correction as data did not share equal standard deviations.

Candida CFU/mL was significantly increased in samples from patients with RVVC to 2.3×10^4 CFU/mL from 2×10^3 CFU/mL in healthy patients ($P < 0.01$) (Figure 3.4A). Molecular analysis allowed for inclusion of *Candida* which are viable but not culturable by quantification of total *Candida* DNA in swab samples. When assessed by real-time qPCR, a similar significant increase of around 1.3-log was observed in patients with RVVC to 3.8×10^5 CFE/mL ($P < 0.05$) (Figure 3.4B).

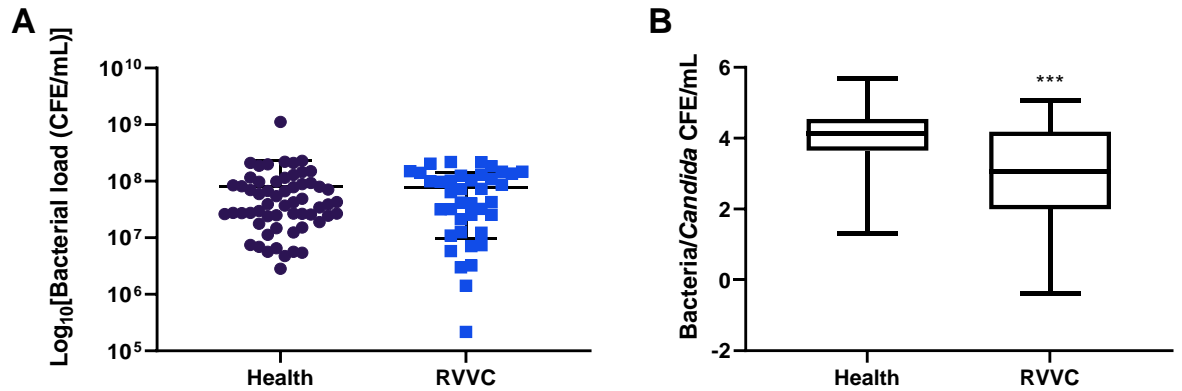


Figure 3.5: Bacterial load remains unchanged in RVVC. Levels of bacterial DNA present in swab samples were assessed molecularly by amplification of the 16S rRNA region (A); Correlation between bacterial and fungal burden was also observed (B). Data represents the mean \pm SD (***, $P < 0.001$); Statistical significance was calculated using unpaired t-tests with Welch's correction as data did not share equal standard deviations.

Bacterial load was found to be comparable between the two disease states and correlation of bacterial-*Candida* levels confirmed the observed increase in fungal load in RVVC samples (Figure 3.5). This data suggests that increased levels of *Candida* could be a contributing factor to disease pathology and may be an indicator for RVVC onset and subsequent recurrence.

As these are clinical samples, it was hypothesised that patient behaviour could influence the observed fungal load. To investigate this, culturable and detectable *Candida* levels were observed with respect to how long patients had suffered from RVVC (Figure 3.6 and Figure 3.7).

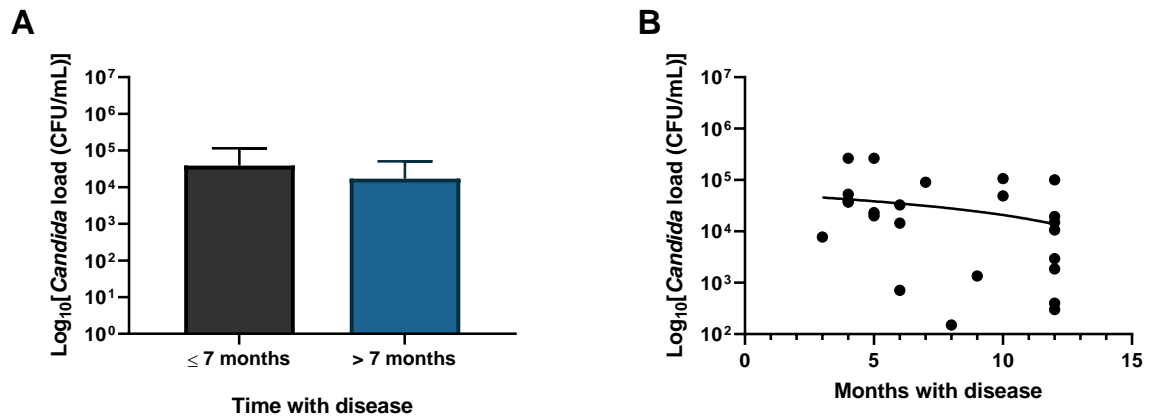


Figure 3.6: Correlation of culturable *Candida* load with length of time with RVVC. Comparison of culturable *Candida* load in patients with RVVC (A) and linear regression analysis of *Candida* load over time (B). Data represents mean + SD.

Although a slight reduction from $\sim 1 \times 10^5$ to 1×10^4 CFU/CFE/mL is observed in patients with disease for over 7 months, this was not significant for either culturable ($P=0.28$) or detectable ($P=0.08$) *Candida* levels (Figure 3.6A and 3.7A, respectively). Regression analysis confirmed this showing a slight decrease in *Candida* load over time (Figure 3.6B and 3.7B). This data suggests that fungal burden is not necessarily a key factor in disease progression, and it may be more important to investigate factors such as biofilm formation and transcriptional changes.

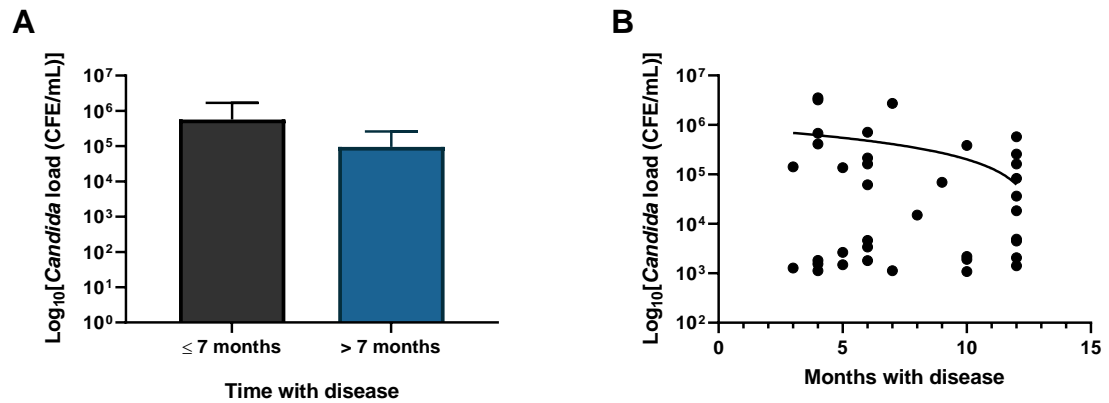


Figure 3.7: Correlation of detectable *Candida* load with length of time with RVVC. Comparison of detectable *Candida* load in patients with RVVC (A) and linear regression analysis of *Candida* load over time (B). Data represents mean + SD.

To investigate whether antifungal treatment of patients could have affected *Candida* load, the length of time since patients had been treated was compared with levels of culturable and detectable *Candida* (Figure 3.8 and Figure 3.9).

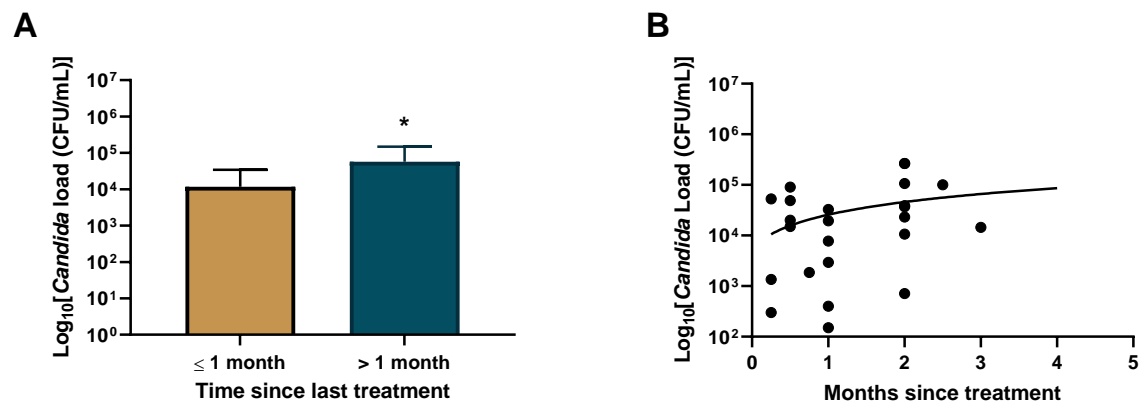


Figure 3.8: Correlation of culturable *Candida* load with treatment time. Culturable *Candida* load was analysed with respect to the length of time since patients had received RVVC treatment (A). Linear regression of *Candida* load against time since treatment (B). Data represents mean + SD (*, $P < 0.05$), statistical analysis was performed using unpaired t-tests.

A significantly decrease of around 1-log of culturable fungal load was observed in patients who had received treatment within one month of sample collection compared to those treated less frequently ($P < 0.05$) (Figure 3.8A). Although not significant when measured by qPCR, a similar trend was observed ($P = 0.23$) (Figure 3.9A). Additionally, regression analysis confirmed this showing a positive correlation between *Candida* load and the number of months since treatment was given (Figure 3.8B and 3.9B).

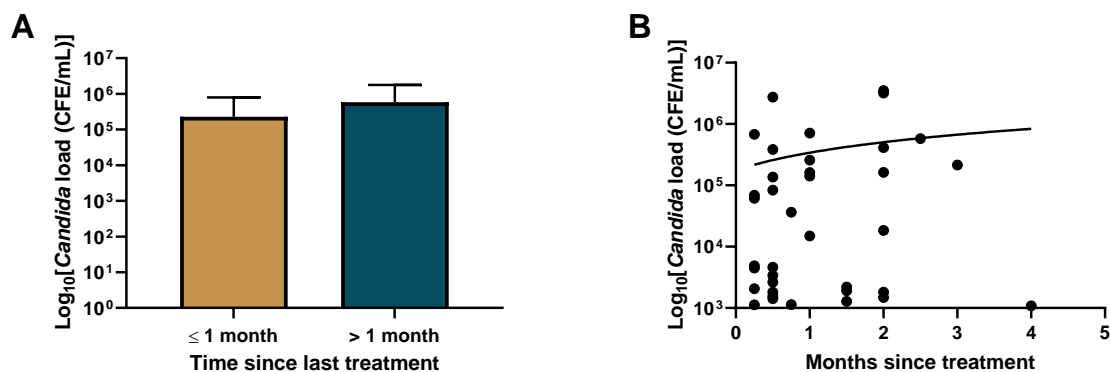


Figure 3.9: Correlation of detectable *Candida* load with treatment time. Detectable *Candida* load was analysed with respect to the length of time since patients had received RVVC treatment (A). Linear regression of *Candida* load against time since treatment (B). Data represents mean + SD (*, $P < 0.05$), statistical analysis was performed using unpaired t-tests.

3.3.3 Detection of inflammatory biomarkers

A subset of samples was selected for Olink analysis to assess levels of inflammatory biomarkers present in CVL supernatant ($n = 20$). The top ten samples with highest levels of detectable *Candida* were selected from each patient cohort for this analysis. Proteomic analysis allowed for the identification of potential specific biomarkers associated with RVVC (Figure 3.10).

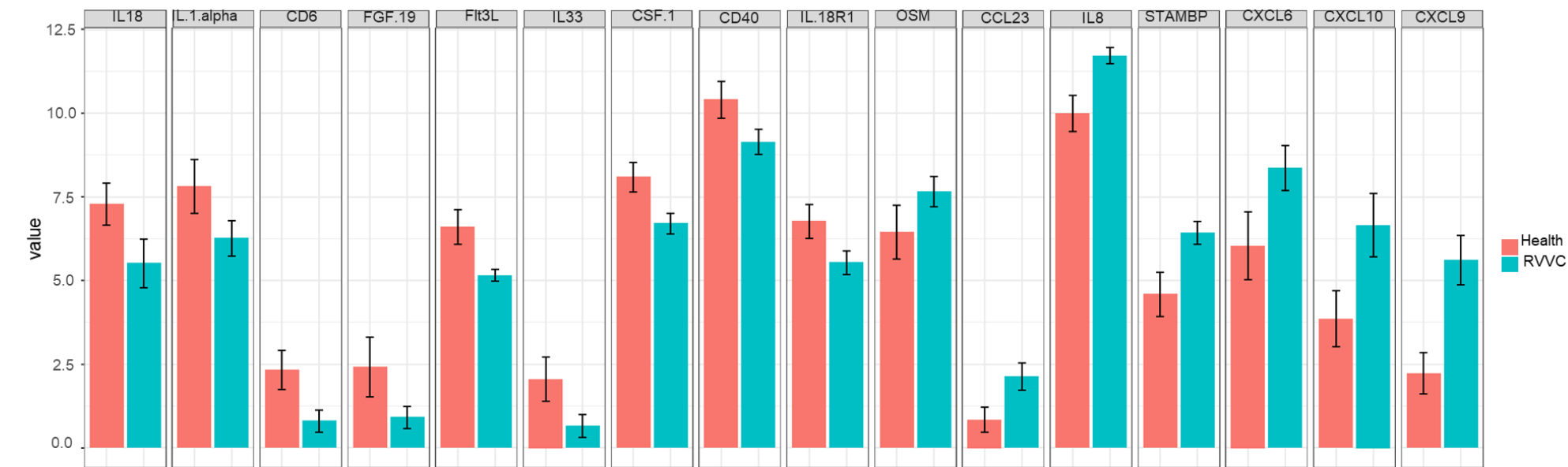


Figure 3.10: Inflammatory profile of health and RVVC.
Proteomic analysis was performed on lavage supernatant of healthy (n=10) and RVVC (n=10) samples. Bar plot representing proteins, which are significantly differentially expressed between health and RVVC. T-tests using FDR-corrected P-values <0.05 were considered to be significant.

Proteomic analysis revealed significantly higher levels of inflammatory biomarkers in samples from patients with RVVC including a series of chemokines: IL-8, CCL-23, CXCL6, CXCL9 and CXCL10 (Figure 3.10). Further, some of the assessed inflammatory biomarkers were found to be higher in health compared with RVVC including IL-18, IL-33, and CSF-1. This elevation in healthy controls may be due to underlying autoimmune or inflammatory diseases which were not controlled for in this study. For example, IL-18 is associated with various diseases including influenza virus infections and Crohn's disease (Yasuda et al., 2019), elevated IL-33 has been linked to asthma and atopic dermatitis (Miller, 2011), and elevated CSF-1 has been associated with general inflammation (Hume and MacDonald, 2012).

Multivariate analysis by Principal Component Analysis (PCA) was performed to identify clusters of inflammatory profiles between health and RVVC (Figure 3.11).

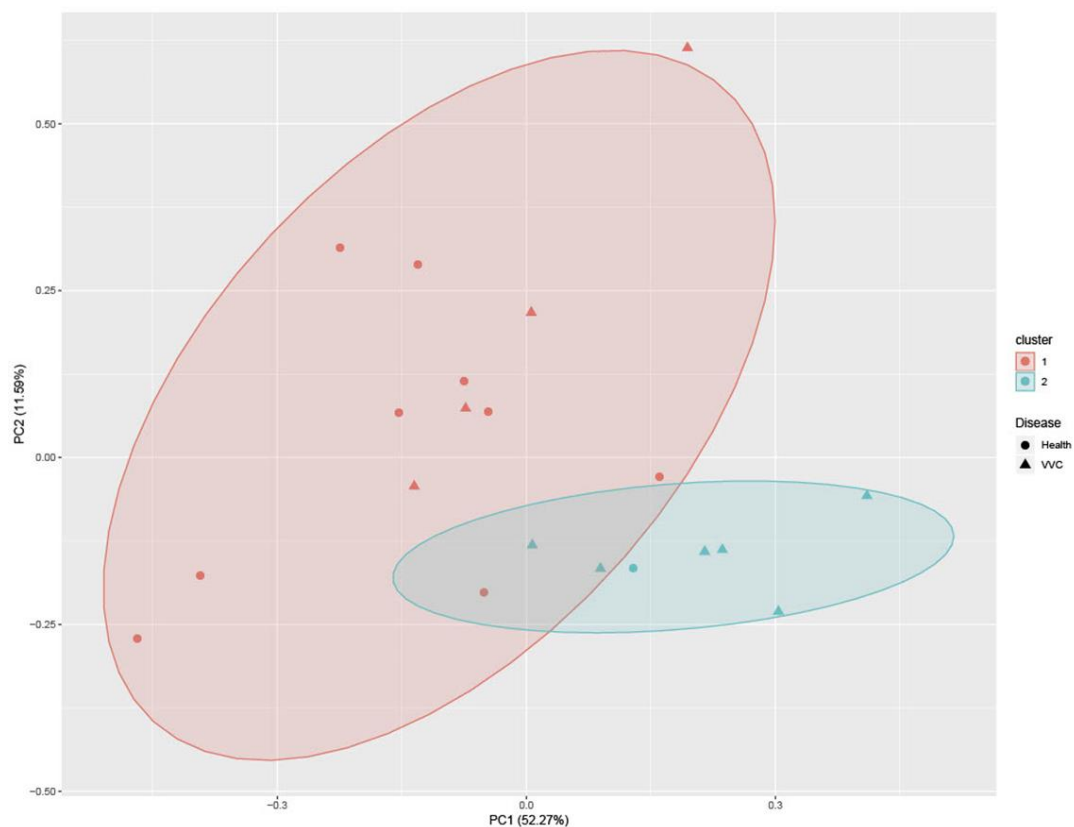


Figure 3.11: Clustering of inflammatory profiles of health and RVVC. Principal Component analysis (PCA) with ellipse showing clusters of biomarkers associated with health or RVVC (B). PC1 shows the largest variance (52.27%) along the x-axis and PC2 displays the second largest variance (11.59%) on the y-axis.

This analysis revealed two major clusters with cluster 1 comprising predominantly healthy samples and cluster 2 containing mainly those from patients with RVVC. Clear separation of biomarker clusters was observed with some overlap between the two clusters.

To further investigate this pattern of inflammatory chemokine upregulation in RVVC observed here, ELISAs were performed on the remaining CVL supernatants of all samples to assess levels of CXCL9, CXCL10 and IL-8 (Figure 3.12).

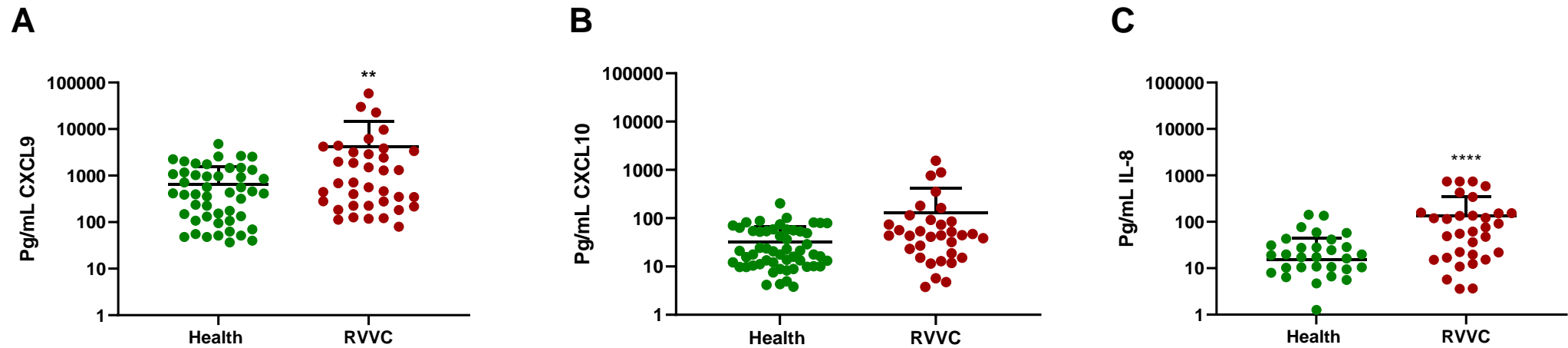


Figure 3.12: Pro-inflammatory cytokines are elevated in patients with RVVC.

Vaginal lavage supernatant samples were assessed by ELISA for levels of the inflammatory chemokines, CXCL9 (A), CXCL10 (B) and IL-8 (C) (n=98). ELISAs were performed in triplicate (**, $P < 0.01$; ****, $P < 0.0001$); Statistical significance was measured by Mann Whitney tests as data was not normally distributed. Data represents mean \pm SD.

Analysis of levels of inflammatory chemokines in all CVL supernatants revealed elevated levels of all assessed biomarkers in RVVC compared with health (Figure 3.12). Around a 1-log increase in chemokine levels was observed in RVVC for all chemokines assessed: CXCL9 ($P<0.01$), CXCL10 ($P=0.169$) and IL-8 ($P<0.0001$).

Additionally, chemokine levels were observed with respect to the presence or absence of culturable *Candida* in the samples (Figure 3.13).

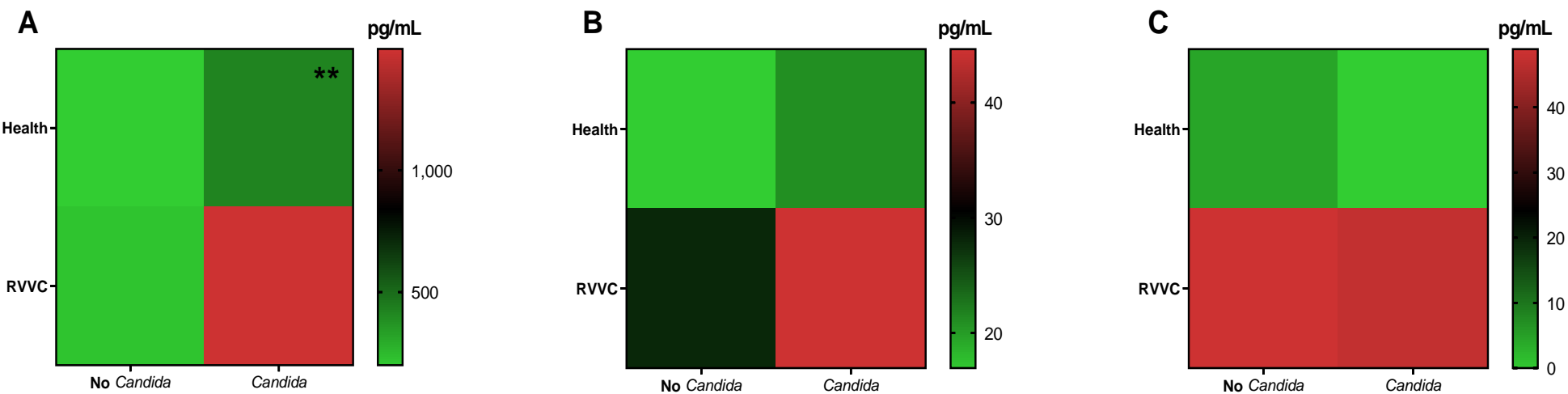


Figure 3.13: The presence of *Candida* elevates levels of pro-inflammatory chemokines in RVVC lavage samples.

Heatmaps were constructed to observe levels of (A) CXCL9, (B) CXCL10 and (C) IL-8 with respect to presence/absence of culturable *Candida*. (**, $P < 0.01$); Statistical significance compared chemokine levels in RVVC in the presence and absence of *Candida* by Mann Whitney tests as data was not normally distributed.

Interestingly, this analysis revealed a pattern by which CXCL9 and CXCL10 chemokine levels in RVVC are elevated further when *Candida* is culturable in the sample. This was significant for CXCL9 ($P < 0.01$), but not for CXCL10 ($P = 0.1$). This trend was not observed in IL-8, where observed chemokine levels were comparable in the presence and absence of *Candida*.

3.3.4 Assessing *Candida* biofilm formation in clinical isolates

One common cause of failed clinical treatment is the formation of microbial biofilms, although this is highly disputed in RVVC research. To observe evidence of *Candida* aggregation and filamentation in CVL samples, lavage was stained and incubated with CFW before being imaged under a light microscope (Figure 3.14).

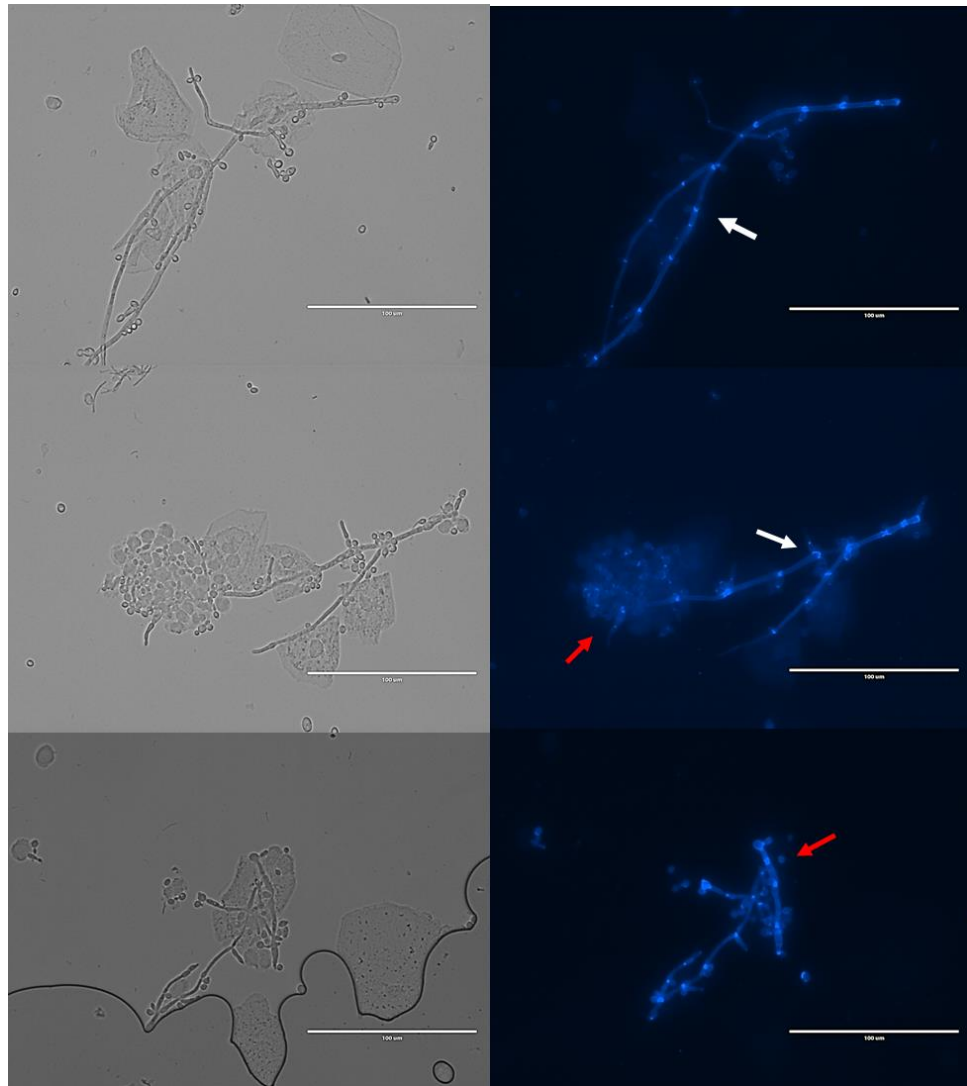


Figure 3.14: *Candida* filamentation and aggregation is present in RVVC lavage. Vaginal lavage was stained with CFW for 1 h at 37°C before fluorescent imaging. Images are representative of *C. albicans* aggregates and hyphae observed in lavage from a patient with a high biofilm forming isolate. White arrows represent pseudo-hyphal/hyphal formation and red arrows depict cell aggregates.

As depicted in Figure 3.14, clear evidence of *C. albicans* hyphal morphogenesis and aggregation can be seen in lavage fluid from a patient with RVVC. This strengthens previous evidence that *Candida* forms biofilms in the vagina during RVVC and may be an underappreciated reservoir for antifungal resistance and failed clinical treatment.

Given the biofilm phenotype observed above, to further assess the potential impact of *Candida* biofilms within our sample subset, we observed the species distribution and biofilm forming capabilities of the vaginal isolates (Figure 3.15). *C. albicans* is the most isolated causative organism in VVC, the importance of non-*albicans* *Candida* species (NACs) should not be underestimated. *Candida* clinical isolates were obtained from lavage samples by culture on *Candida* chromogenic agar. After incubation, a total of 33 isolates were obtained, 9 from healthy individuals and 24 from patients with RVVC. Isolate speciated by chromogenic agar were confirmed to species-level using MALDI-TOF. A large area of controversy in VVC research is the presence of *Candida* biofilms on vaginal epithelium and their contribution to failed clinical treatment. For this reason, following identification, *Candida* clinical isolates were screened for their *in vitro* biofilm forming capabilities (Figure 3.15).

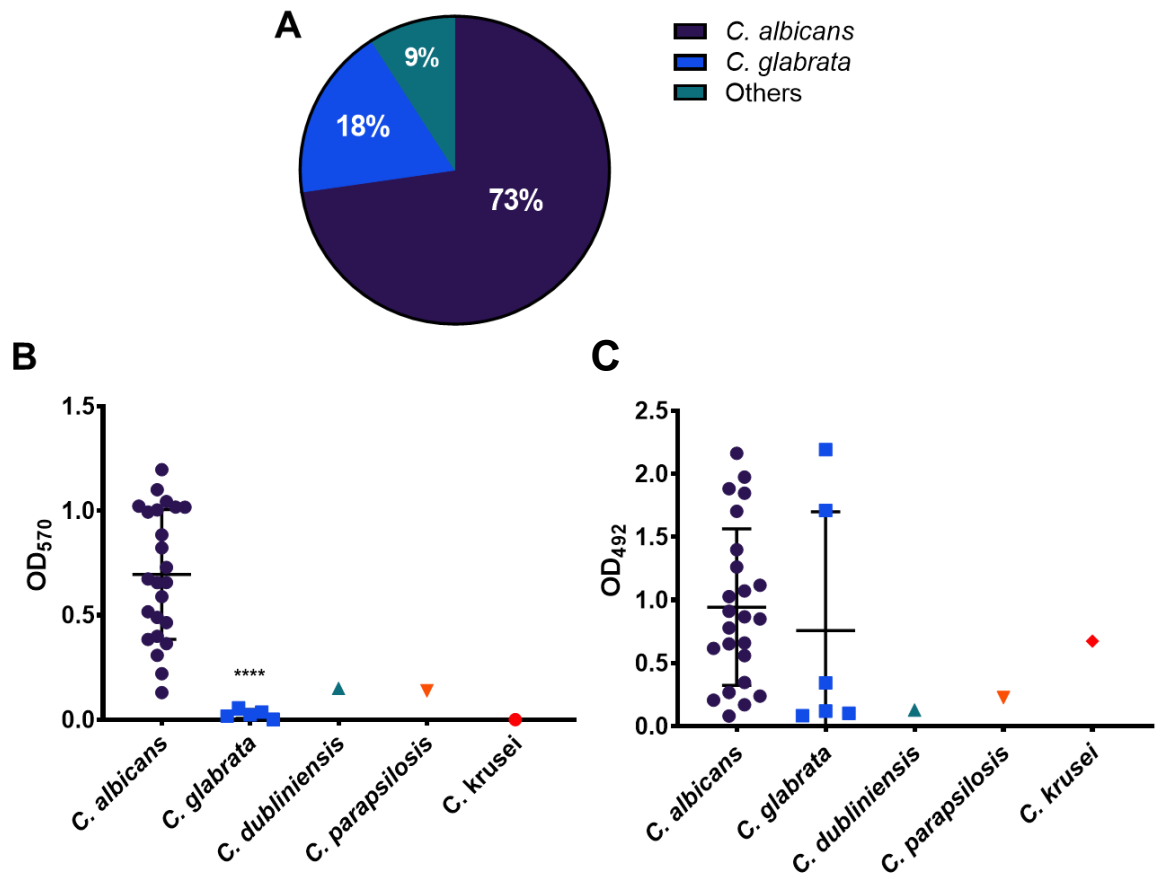


Figure 3.15: Vaginal *Candida* isolates from patients with RVVC are capable of heterogeneous biofilm formation. MALDI-TOF was used to identify each isolate to species level and assess species distribution ('others' are comprised of 1 isolate of: *C. dubliniensis*, *C. parapsilosis* and *C. krusei*) (A). *Candida* isolates from health (n=9) and RVVC (n=24) were assessed for biofilm forming capabilities and metabolic activity by crystal violet staining and XTT assays, respectively (B and C). Data represents the mean \pm SD (****, $P < 0.0001$), statistical analysis was performed using unpaired t-tests.

Consistent with previous RVVC studies, *C. albicans* was found to be the most prevalent *Candida* species isolated within our patient subset. Other NAC species, more commonly associated with complicated VVC, accounted for the remaining 27% (Figure 3.15A). *C. albicans* was capable of forming dense biofilms with clear heterogeneity between isolates. A wide range of OD values between 0.13 and 1.067 was observed for *C. albicans* biofilm formation with a mean OD of 0.695. Little biomass was observed in other species (Figure 3.15B). Similarly, *C. albicans* biofilms displayed the highest variation of metabolic activity amongst RVVC isolates. Metabolic activity of *C. albicans* isolates ranged from an OD of 0.079 and 2.162 with a mean OD of 0.943. Notably, although they did not form robust biofilms, some *C.*

glabrata isolates were highly metabolically active ranging from 0.082 and 2.192 with a mean of 2.11 (Figure 3.15C).

One of the main aims of this study was to determine potential factors contributing to failed clinical treatment of RVVC within our patient subset. To assess antifungal resistance to clinically relevant concentrations (Up to 32 µg/mL) of frontline antifungals such as fluconazole, miconazole and clotrimazole within clinical isolates, MICs were determined for each drug for both planktonic and sessile cells (Table 3.5).

Table 3.5: Sensitivity profiles of *Candida* clinical isolates to azole antifungals.

	pMIC ^{50*} (µg/mL)			pMIC ^{90§} (µg/mL)			sMIC [#] (µg/mL)		
	F	M	C	F	M	C	F	M	C
<i>C. albicans</i> (n= 25)									
SVS007A	1	<0.063	<0.063	>32	>32	>32	>32	8	1
SVS013A	1	<0.063	<0.063	>32	>32	>32	>32	16	16
SVS026A	1	<0.063	<0.063	>32	8	8	16	8	0.25
SVS036A	16	0.5	0.5	>32	16	2	>32	8	16
SVS040A	1	<0.063	<0.063	>32	>32	>32	>32	16	32
SVS043A	1	<0.063	<0.063	>32	>32	>32	16	8	8
SVS051A	2	<0.063	<0.063	>32	>32	>32	32	8	16
SVS002B	1	<0.063	<0.063	>32	>32	32	>32	>32	4
SVS003B	>32	0.25	<0.063	>32	32	32	>32	8	8
SVS006B	1	<0.063	<0.063	>32	>32	>32	>32	8	>32
SVS007B	2	0.5	<0.063	>32	>32	>32	>32	8	>32
SVS008B	0.13	<0.063	<0.063	>32	>32	>32	>32	8	16
SVS009B	2	<0.063	<0.063	32	2	2	>32	16	32
SVS010B	>32	0.25	0.25	>32	16	16	>32	>32	>32
SVS011B	4	<0.063	<0.063	32	4	2	>32	32	32
SVS012B	1	<0.063	<0.063	8	2	2	>32	16	32
SVS015B	2	<0.063	<0.063	>32	16	8	>32	16	32
SVS019B	1	<0.063	<0.063	>32	>32	>32	16	8	1
SVS021B	32	0.13	<0.063	>32	8	8	>32	4	16
SVS023B	1	<0.063	<0.063	>32	16	>32	>32	16	32
SVS026B	2	0.25	0.5	32	8	4	32	8	>32
SVS027B	0.5	<0.063	<0.063	32	4	0.5	>32	32	>32
SVS031B	8	1	<0.063	>32	8	1	>32	8	>32
SVS032B	2	0.5	0.25	>32	8	1	>32	16	>32
SVS034B	4	0.5	0.5	>32	8	2	>32	4	>32
<i>C. glabrata</i> (n=6)									
SVS001A	2	<0.063	<0.063	>32	8	32	>32	16	16
SVS011B	4	<0.063	<0.063	>32	>32	32	>32	32	>32
SVS018B	16	0.125	0.25	>32	0.25	1	>32	>32	>32
SVS022B	0.5	<0.063	<0.063	>32	16	32	>32	16	16
SVS029B	4	<0.063	1	8	0.25	8	>32	>32	>32
SVS033B	2	<0.063	<0.063	16	<0.063	0.25	2	32	16
<i>C. dubliniensis</i> (n=1)									
SVS057A	0.5	<0.063	<0.063	1	<0.063	<0.063	>32	2	4
<i>C. parapsilosis</i> (n=1)									
SVS026A	0.5	<0.063	<0.063	2	16	8	32	4	1
<i>C. krusei</i> (n=1)									
SVS004B	32	8	0.25	>32	32	16	>32	8	4

F, M, C: fluconazole, miconazole and clotrimazole, respectively.

* 50% planktonic minimum inhibitory concentration

§ planktonic minimum inhibitory concentration

50% sessile minimum inhibitory concentration

Antifungal susceptibility testing revealed 50% inhibition of all planktonic isolates at concentrations ≤ 32 $\mu\text{g/mL}$ for fluconazole, with the exception of two *C. albicans* RVVC isolates (SVS002B and SVS010B). Additionally, 50% planktonic inhibition of all isolates was achieved for miconazole and clotrimazole at ≤ 8 $\mu\text{g/mL}$ and ≤ 1 $\mu\text{g/mL}$, respectively. Fluconazole was the least effective treatment against planktonic *Candida* isolates achieving 90% inhibition in just 24% of isolates. Furthermore, 34% and 29% of isolates contained populations of cells which resisted 90% inhibition with miconazole and clotrimazole at concentrations up to 32 $\mu\text{g/mL}$, respectively. Finally, fluconazole was also the least effective antifungal against *Candida* biofilms, achieving 50% inhibition in just 21% of isolates. Miconazole and clotrimazole were more effective against sessile cells, however, 12% and 32% of isolates, respectively, remained viable following treatment.

Finally, to further investigate the potential impact of *Candida* biofilm formation in RVVC, levels of expression of key biofilm-related genes in *C. albicans* were measured by real-time qPCR in a subset of samples (n=20) (Figure 3.16). The selected sample subset used contained the 10 highest levels of *Candida* DNA for each cohort.

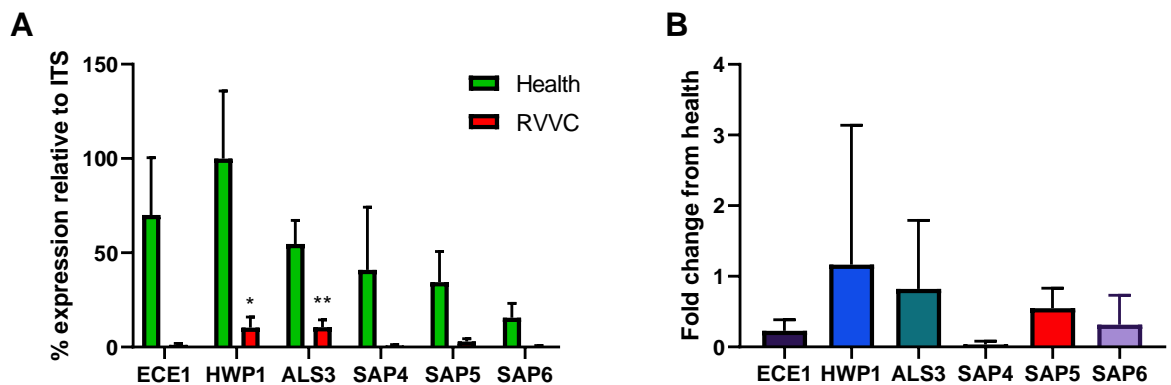


Figure 3.16: *Candida* biofilm-related genes are not up-regulated in lavage from women with RVVC. Total RNA was extracted from patient lavage before being reverse transcribed to cDNA for gene expression analysis. Samples from healthy (n=10) and RVVC (n=10) patients were assessed for expression of key *Candida* biofilm-related genes relative to expression of the *ITS* housekeeping gene. Expression levels were measured by percentage expression (A) and fold change from health (B). Data represents the mean \pm SD (* $P < 0.05$; ** $P < 0.01$), statistical analysis was performed using unpaired t-tests with Welch's correction.

Gene expression analysis was measured as both percentage expression relative to the housekeeping *ITS* gene (Figure 3.16A) and as fold change in RVVC from health (Figure 3.16B). Genes assessed, including *ECE1* and *HWP1*, were found to have similar levels of expression to the housekeeping gene in healthy samples with mean expression levels of 70% and 99%, respectively. All other genes were downregulated in healthy samples with <55% expression relative to *ITS*. In RVVC samples, *ECE1*, *SAP4*, *SAP5* and *SAP6* were undetectable. Significantly lower levels of expression of *HWP1* and *ALS3* were observed compared with healthy controls ($P < 0.05$, $P < 0.01$, respectively). This is clearly reflected in Figure 3.16B where the fold change of *HWP1* and *ALS3* relative to expression in healthy samples is around 1-fold, whereas the rest of the genes fall below 0.55-fold.

3.4 Discussion

Despite surprisingly high numbers of cases around the globe annually, VVC and subsequent RVVC remains largely understudied in the field of women's health. For this reason, further research to understand the onset and progression of disease is paramount for the development of much needed future therapeutics. This chapter evaluated a panel of clinical samples from healthy women and those with RVVC. This was achieved through a variety of microbiological, molecular, and proteomic approaches. Women were recruited for the study if they had visited their GP surgery with complaint of VVC four or more times within one year or were self-referred. Additionally, healthy women attending clinics for an IUD to be fitted were recruited as healthy controls for the study.

The estimated vaginal commensal carriage rate of *Candida* is 33%. The presence of *Candida* was observed by both culture and by qPCR which observed 15% and 60% of healthy and RVVC samples had culturable *Candida*, respectively. All but 2 samples were positive by qPCR. In vaginitis caused by bacterial vaginosis (BV), dysbiosis of the vaginal microbiome is thought to result in an increase in pH (pH >5) due to a reduction in lactic acid bacteria, particularly *Lactobacillus* species (Wilson, 2004). Conversely, the pH during VVC is often reported to be normal (pH 4-4.5) (Linhares et al., 2011). Here it is shown that patients suffering from RVVC had comparable vaginal lavage pH levels with those from healthy patients. The pH range varied from around pH 4 to 7 and may simply vary by patient. It is thought that a low vaginal pH contributes to a eubiotic environment due to its inhibitory properties to many pathogens including *Candida* (O'Hanlon et al., 2011, Gong et al., 2014, Zangl et al., 2019). Low pH environments have been shown to be fungistatic, inhibiting *Candida* hyphal morphogenesis and biofilm formation (Parolin et al., 2015). Despite the evidence, the pH in this study was found to be non-specific to the disease status of patients. A likely explanation for this outcome is the method of collection of CVL samples; unlike swab samples or the direct collection of vaginal fluid, CVL are collected by pooling and removing sterile water (pH 7) from the vagina of the patient. It is likely that this method of collection increased or neutralised the acidic pH of the vaginal fluid in this study.

Although authors have suggested that an increased fungal load may be correlated with symptomatic VVC, very few studies have investigated *Candida* counts in their sample cohorts (Hopwood et al., 1988). To investigate the influence of fungal load, levels of *Candida* in swab samples were measured by plate dilution and molecularly. This investigation found significantly higher levels of 1 or 2-log of *Candida* in samples from patients with RVVC compared with healthy patients. Increases in *Candida* culture positive samples have been reported previously in RVVC and is confirmatory of disease state (Bauters et al., 2002). However, these authors did not measure *Candida* load by cell count, only determining whether patients were culture negative or positive. Despite the applications of real-time qPCR to accurately quantify *Candida* load in patient samples, it is not FDA-approved, and culture remains the “gold standard” for VVC diagnosis (Chatzivasileiou and Vyzantiadis, 2019). Bacterial levels in each cohort were found to be comparable, suggesting *Candida* does not replace the vaginal microbiome during disease, but may instead impact the diverse bacterial composition resulting in symptoms of disease. Patient questionnaires were used to collect data on patient’s disease and treatment histories and this allowed for assessment of fungal burden data with respect to disease severity and treatment regimens. This data revealed a positive correlation with increasing levels of *Candida* since antifungal treatment. This suggests antifungal treatment may temporarily decrease levels of *Candida* and alleviate symptoms of RVVC, however without effective clearing and with the presence of resistant communities of cells *Candida* is able to regrow and cause recurrence of symptoms. This data also revealed a slight reduction in *Candida* levels over time with disease. Future studies may wish to use larger sample groups to determine whether this is consistent. It is also important to consider that these are clinical samples from women regularly attending sexual health clinics, and those who have suffered from RVVC for longer are more likely to be receiving antifungal treatment. There is clear lack of studies investigating fungal load of patients with RVVC compared with healthy controls in the literature. In fact, to our knowledge this is the first study reporting yeast counts during RVVC since 1988. Further investigation could elucidate whether an “infection threshold” exists for RVVC and could improve diagnostic practices through measuring and maintaining *Candida* levels within the vagina.

VVC is an inflammatory disorder, characterised by extensive mucosal inflammation of the vulva as a result of leukocyte influx in response to hyphal invasion of local epithelium and expression of *Candida* virulence factors (Rosati et al., 2020). This study found a pattern of elevated pro-inflammatory cytokine levels in patients with RVVC. Similar trends have been shown *in vitro* when treating both vaginal epithelial cells and murine models of VVC with candidalysin, a crucial virulence factor in *Candida* (Richardson et al., 2018). Many of the inflammatory biomarkers found to be upregulated in RVVC samples have been previously described in inflammatory diseases such as psoriasis, rheumatoid arthritis and hepatitis (Abji et al., 2016, Kuan et al., 2010, Zeremski et al., 2011). Data reported in this chapter also reports elevated levels of pro-inflammatory cytokines in patients with culturable *Candida*. This trend was not seen when assessing IL-8 levels, however, this biomarker is often elevated due to non-specific inflammation (Schroder, 1992).

Consistent with recent epidemiological reports, >70% of isolates from patients were *C. albicans* with the remainder comprised primarily of *C. glabrata*. These results are also consistent with the recently reported increase in NAC species in VVC. Although it has been historically reported that *C. albicans* is the causative organism in up to 90% of VVC episodes, recent evidence has suggested a higher prevalence of NAC in the community. It is possible that this assumption that almost all cases are due to *C. albicans* has led to inappropriate treatment, driving higher rates of NAC RVVC. The clinical significance of *C. glabrata* and, to a lesser extent *C. krusei* as it is rarely the causative organism of VVC, should not be underestimated due to their intrinsic resistance to azole antifungals (Singh et al., 2002). The prevalence of NAC species is thought to increase with each recurrent episode of RVVC. One study aiming to quantify the rate of increase estimated a 10% increase in NAC species prevalence with each subsequent VVC episode (Sobel, 2002). As all patients in this study presented with RVVC, this is a potential explanation for the high occurrence of NAC species seen here.

Though *C. albicans* has been shown to form biofilms on vaginal mucosa and *Candida* clinical isolates shown to form biofilms *in vitro* (Harriott et al., 2010b, Sherry et al., 2017, Swidsinski et al., 2019), the presence of biofilms during RVVC is still disputed. Data in this chapter shows clear evidence of *C. albicans* filamentation and

aggregation in lavage fluid from a patient with RVVC that was visualised fluorescently. Furthermore, this study observed heterogeneous biofilm formation in *C. albicans* isolates as well as highly metabolically active *C. albicans* and *C. glabrata* biofilms. Further, a recent study found both wild type and clinical isolates of *C. albicans* are capable of biofilm formation and epithelial infiltration of vaginal epithelium using the murine model developed by Harriott et al, 2010 (Wu et al., 2020, Harriott et al., 2010b). This data strengthens the argument that *Candida* biofilm formation within the vagina during VVC should not be discounted as a potential cause of failed clinical treatment as suggested by some authors (Swidsinski et al., 2019, Sobel et al., 1998)

Antifungal susceptibility testing found significant levels of resistance of *Candida* clinical isolate biofilms to frontline antifungals used to treat VVC. The significance of antifungal resistance in RVVC has been disputed, with one study showing planktonic susceptibility of 117 *C. albicans* isolates to various prolonged azole treatments (Lynch et al., 1996). This study failed to consider substantially increased antifungal resistance observed in sessile cells compared to their planktonic counterparts. Biofilm resistance to these azoles was high, as expected, however interestingly, total killing of planktonic cells was achieved in only 24%, 34% and 29% of isolates treated with up to 32 µg/mL of fluconazole, miconazole and clotrimazole, respectively. These communities of resistant “persister cells” are another potential reservoir for failed clinical treatment and have been described in previous studies. One study using VVC clinical isolates recovered up to 3% of cells from biotic biofilms which persisted through overnight treatment with 100 µg/mL amphotericin B (Wu et al., 2020). This highlights an important caveat in RVVC research which could lead to more appropriate treatment for women with biofilm-forming, antifungal resistant isolates. More recently, studies have focussed on the potential efficacy of probiotic treatment and the prospect of a vaccine against VVC (Schmidt et al., 2012, Xie et al., 2017). The potential VVC vaccine, NDV-3, has been shown to significantly reduce fungal burden in a murine model and further research could result in this becoming a safe and effective treatment to prevent VVC and recurrent episodes (Ibrahim et al., 2013). Recently, a new antifungal was FDA-approved for the treatment of vaginal yeast infections, Ibrexafungerp. This drug is the first of the enfumafungin-derived triterpenoid family of antifungals as well as the first oral (1-3)-

β -D-glucan synthase inhibitor (GSIs) (Ghannoum et al., 2020). This drug has been shown to be effective against a range of *Candida* isolates, including those displaying resistance to azoles and echinocandins (Jimenez-Ortigosa et al., 2014). Additionally, it has shown efficacy against *C. albicans* and *C. glabrata* biofilms *in vitro*, with lower MICs than those seen with fluconazole (Marcos-Zambrano et al., 2017). These are very promising results and with granted FDA-approval this drug has potential to greatly improve treatment options and reduce recurrence rates of RVVC.

To further investigate biofilm formation in RVVC, expression of key biofilm-related genes was assessed. Expression analysis found relatively low levels of expression in RVVC samples for all genes assessed compared with the housekeeping *ITS* gene. Despite this, data in this chapter is one of the few reports of detectable levels of biofilm-related genes in clinical samples of RVVC. One study quantifying *SAP* expression from vaginal swabs reported *SAP1-10* expression to be unchanged during vaginitis with the exception of *SAP5*, which was significantly upregulated (Naglik et al., 2008). Data from this chapter suggests, although *Candida* biofilms are present during RVVC, very low levels of biofilm-related gene expression are detectable from patient lavage. One possibility for this result is the length of time these clinical samples had been stored and number of freeze-thaw cycles prior to RNA extraction. Future studies utilising whole-genome transcriptome sequencing are required to fully elucidate the significance of biofilm-related gene expression in RVVC.

Chapter findings

- Data from this chapter demonstrated that, though unchanged throughout disease progression, an increased *Candida* load may be indicative of RVVC. Further, an increase in pro-inflammatory cytokine levels in lavage fluid may strengthen diagnosis.
- There is potential for failure to eradicate *Candida* following clinical treatment as although *Candida* load is reduced, resistant communities of cells persist and can re-establish RVVC. Further, data in this chapter is consistent with recent reports of an increase in the prevalence of NAC in RVVC. These clinical isolates display resistance to clinically relevant concentrations of frontline azole treatments. Collectively, these data are indicators of *Candida* biofilm formation during RVVC.
- Through biomass and viability assays of clinical isolates, imaging of lavage and detection of biofilm-related gene expression in lavage, there is clear evidence of biofilm formation amongst RVVC clinical isolates. As such, it is crucial to consider *Candida* biofilm formation, and the repercussions this may have, when making clinical decisions for treatment of RVVC.

4 The importance of *Lactobacillus* species in RVVC

4.1 Introduction

The development of molecular methods of bacterial identification like 16S ribosomal RNA (rRNA) sequencing has dramatically improved our ability to identify and understand organisms within niches of the human body. The 16S rRNA gene is present in all bacteria and its combination of conserved and hypervariable regions make it an ideal candidate for identification of different bacteria via polymerase chain reaction (PCR) (Wilson et al., 1990). This becomes more important in less diverse and anaerobic niches where many of the microorganisms present are unculturable. The vaginal microbiome is somewhat unique in that a 'healthy' microbiome is generally associated with a less diverse community of microbes, predominantly lactobacilli, namely; *L. crispatus*, *L. iners*, *L. gasseri* and *L. jensenii* (Ravel et al., 2011a). Dysbiosis of the vaginal microbiome therefore results in an increase in species diversity, with fewer lactobacilli and greater levels of pathogenic anaerobes such as *Gardnerella* and *Prevotella* species (Liu et al., 2013b, Jian et al., 2015). In VVC, although unconfirmed, disease onset is thought to be attributed this loss of lactobacilli and a concomitant overgrowth of commensal *Candida* species (Bradford et al., 2017). This facilitates a pathogenic shift in *Candida* allowing hyphal morphogenesis, cell damage and activation of the immune response, causing symptoms of vaginitis.

In healthy women, lactic acid bacteria (LAB) are thought to be responsible for maintaining a homeostatic microbiome by inhibiting growth and adhesion of other microbes via the production of secreted metabolites such as lactic acid, biosurfactants, bacteriocins and hydrogen peroxide (H₂O₂) (Redondo-Lopez et al., 1990, Jeavons, 2003). Of the four most common *Lactobacillus* species in the healthy microbiome, *L. crispatus*, *L. gasseri* and *L. jensenii* produce both D- and L-isomers of lactic acid as well as H₂O₂ (Reid, 2018, van der Veer et al., 2019). Although both isomers afford protection from pathogenic invasion, the L-lactic acid isomer has been extensively studied for its ability to lower vaginal pH, elicit anti-inflammatory responses and inhibit microbial colonisation (Hearps et al., 2017, O'Hanlon et al., 2013). Conversely, lactic acid lowers the pH of the vaginal environment which could result in *Candida* stress responses leading to hyphae formation and release of virulence factors. This was shown by Beyer and colleagues (Beyer et al., 2018a) where the MAP kinase CgHog1 of *C. glabrata* was upregulated in response to

clinically relevant concentrations of lactic acid. CgHog1 increases the ability of *C. glabrata* to persist within the body, suggesting this could contribute to severity of RVVC.

Unlike other vaginal diseases such as bacterial vaginosis (BV) and *Chlamydia trachomatis*, microbiome studies have not been able to determine a distinct profile of disease for RVVC compared to health (Ceccarani et al., 2019). The widely accepted hypothesis of RVVC pathology is a reduction in *Lactobacillus*, however, studies have observed mixed results when investigating this. Some studies have identified reduced levels of *Lactobacillus* during VVC/RVVC (McClelland et al., 2009, Liu et al., 2013b), whereas other authors report similarly high levels of *Lactobacillus* found in both health and RVVC (Macklaim et al., 2015, Zhou et al., 2009a). For this reason, the functional capacity of lactobacilli to inhibit pathogens and prevent vaginal dysbiosis has been questioned. A common feature of many microbiome studies is a change in the *Lactobacillus* population at species-level. These studies have identified a consistent reduction in *L. crispatus* levels in RVVC concomitant with an increase in rates of *L. iners* (Ravel et al., 2011a, Ceccarani et al., 2019). With this, it is important not to discount the potential influence of *Lactobacillus* in RVVC pathology and re-establishing a healthy microbiome. Instead, studies should focus on the intimate interactions between *Candida* and these *Lactobacillus* species in order to determine how they impact states of health and disease.

Hypotheses and aims

Potential interkingdom interactions occurring as a result of *Candida* overgrowth in the vagina during disease onset is vastly understudied in RVVC research. It is largely unknown how this increase in fungal load may influence the diversity and microbial interactions within the vaginal microbiome. Identifying mechanisms by which microbes of the healthy vaginal microbiome may attempt to maintain homeostasis, as well as those leading to adverse interactions in disease, could elucidate potential probiotic therapeutic targets, expanding and improving future treatment options. Therefore, this chapter aimed to:

- Compare bacterial taxa identification at family and genus-level using OTU and ASV analysis pipelines.
- Identify how *Candida* overgrowth in the vagina affects the bacterial diversity and composition of the microbiome.
- Compare short-read Illumina sequencing with sequencing of ultra-long reads using the Oxford Nanopore Technologies MinION platform.
- Utilise patient metadata in an attempt to identify how the microbiome is affected over time, antifungal treatment regimens and what influence contraception may have during RVVC.

It is hypothesized that lactic-acid bacteria associated with inhibition of pathogens will be reduced in RVVC compared with health. Additionally, it is expected that differences in disease-associated microbiome profiles will be seen with respect to patient metadata collected at the time of sampling.

Work from this chapter has been presented at the following workshops/conferences:

Eurobiofilms Conference, September 2019, Glasgow, UK

Federation of Infection Societies Conference, November 2019, Edinburgh, UK

Work from this chapter has been published in mSystems:

McKloud E, Delaney C, Sherry L, Kean R, Williams S, Metcalfe R, Thomas R, Richardson R, Gerasimidis K, Nile CJ, Williams C, Ramage G. Recurrent Vulvovaginal Candidiasis: a Dynamic Interkingdom Biofilm Disease of *Candida* and *Lactobacillus*. *mSystems*. 2021 Aug 10:e0062221. doi: 10.1128/mSystems.00622-21.

4.2 Materials and methods

4.2.1 Amplification of the 16S V4 region for Illumina sequencing

Sequencing of the 16S rRNA gene was performed to determine the bacterial communities present in health and RVVC. For this analysis, DNA from swab samples collected as described in section 3.2.3 of this thesis were used. The 16S V4 region of DNA was amplified using a 7500 Real-Time PCR System (Applied Biosystems, UK). The 25µl reaction was prepared by addition of 2µl of DNA to a master mix of 12.5µl NEBNext® Ultra™ II Q5® Master Mix (New England Biolabs, UK), 6.5µl nuclease-free water, 1µl of forward/reverse primers and 2µl of bovine serum albumin (BSA, 10mg/mL). Amplification of the V4 region was achieved using the same 16S rRNA forward primer in each sample (AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMG CCGCGGTAA) with unique fusion Golay adaptors barcoded on the reverse strand (Quince et al., 2015). Samples were then amplified using the following thermal profile: An initial denaturation at 95°C for 5 min followed by 30 cycles of 98°C for 20 s, 60°C for 15 s to anneal and 72°C for 40 s for extension and a final extension step of 72°C for 1 min. Reactions were performed in triplicate, on one occasion and included no template controls (NTC)

4.2.2 Isolation and purification of DNA.

To separate DNA bands for excision of the 16S rRNA, a 1% agarose gel was prepared in 1X Tris-acetate-EDTA (TAE) buffer (w/v). Agarose was weighed before being dissolved in 1X TAE buffer in a microwave. Once cool, 25µl of SyberSafe DNA stain (1µl/10mL) (Invitrogen, Paisley, UK) was added and the solution poured into a gel tray. The gel was allowed to set for 20 min and the gel tank was assembled according to manufacturer's instructions (Anachem, Leicester, UK). The gel was then placed into the tank and covered with 1X TAE buffer. Loading dye (6X) was added to each sample before 75µl was loaded in to each well. The gel was then run for 1 h at 100V before being visualised under UV light.

DNA bands observed at 378bp were excised from the gel using a sterile scalpel and transferred to a RNase-free tube. DNA was extracted from the agarose gel using the Zymoclean Gel DNA Recovery Kit (Cambridge Bioscience, UK) following

manufacturer's instructions. Each tube was weighed and 3 volumes (3x w/v) of agarose dissolving buffer (ADB) was added to the DNA fragment and incubated at 50°C for 10 min until completely dissolved. The melted agarose was loaded on to a Zymo-spin column in a collection tube and centrifuged at 13,000rpm for 30 s. The flow-through was discarded and 200µl of DNA wash buffer added to the column before being centrifuged for another 30 s at 13,000rpm. The wash step was repeated, and the column transferred to a sterile microfuge tube. To elute the DNA, 12µl of elution buffer was added directly to the column matrix and centrifuged for 1 min at 13,000rpm.

4.2.3 Pooling DNA library for sequencing and data analysis pipeline.

Bacterial 16S rRNA concentrations were quantified using the high sensitivity fluorometric Invitrogen Qubit® assay, following manufacturer's instructions (ThermoScientific, Loughborough, UK). For library preparation, the concentration of the amplicons for each sample was standardised to 5 ng/mL in nuclease free water. Finally, 2µl of each sample was transferred to a single DNA low-bind Eppendorf tube, forming the final amplicon pool. The 16S rRNA V4 region was then amplified using the Illumina MiSeq sequencing platform (Edinburgh Genomics) using 2x250bp paired-end reads (University of Edinburgh, 2021).

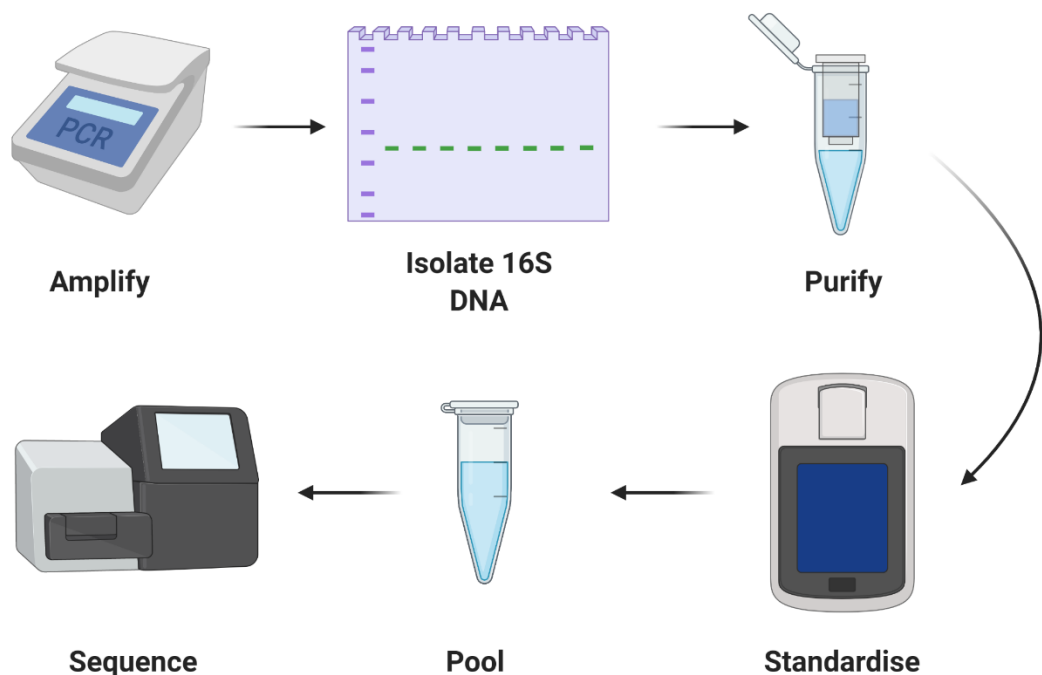


Figure 4.1: Experimental design for 16S rRNA microbiome sequencing using Illumina MiSeq sequencing platform. Created with BioRender.com.

QC of reads was performed within the R package DADA2 (v1.14.1). Reads were filtered and trimmed using standard error rates before reads were denoised, merged and chimeric sequences removed. Amplicon sequence variants (ASV) were annotated by the silva 132 16s rRNA database with an exact match to the ASV. Dr Christopher Delaney (University of Glasgow) performed quality control, sequence trimming and read alignment and enumeration. ASV and taxonomic assignments were utilised for downstream data analysis.

4.2.4 Amplification of DNA for Nanopore sequencing

Samples were prepared for sequencing using the Oxford Nanopore Technologies (ONT) MinION® platform using the 16S Barcoding Kit 1-24 (Oxford, UK), with some modifications. Previously extracted DNA from swab samples was diluted in nuclease-free water to a concentration of 15ng/μl. For each sample a master mix was prepared containing 5μl nuclease-free water, 25μl LongAmp Hot Start Taq 2X Master Mix (New England Biolabs) and 10μl input DNA (150ng). To each sample, 10μl of universal 16S barcodes were added: 27F: AGAGTTTGATCMTGGCTCAG and 1492R: CGGTTACCTTGTTACGACTT to amplify the full 16S region. Each primer pair includes a unique barcode sequence which allows for sample identification following sequencing. The 16S region was then amplified using the following thermal profile: Initial denaturation for 1 min at 95°C then 30 cycles of denaturation for 20s at 95°C, annealing for 30s at 55°C and extension for 2 mins at 65°C, followed by a final extension step for 5 mins at 65°C.

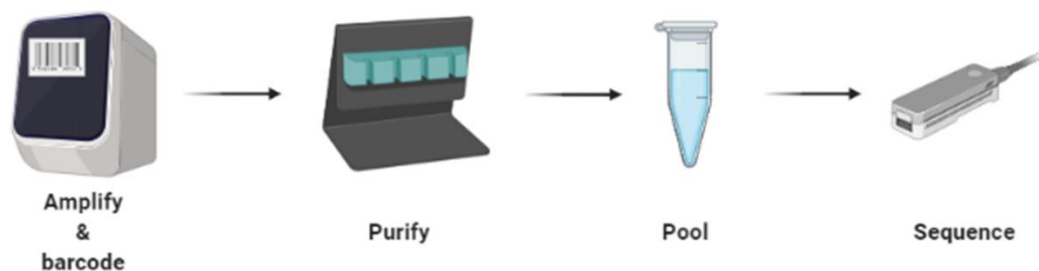


Figure 4.2: Experimental design for 16S rRNA microbiome sequencing using Nanopore MinION® sequencing platform. Created with BioRender.com.

4.2.5 Purification and pooling of DNA libraries.

To clean the DNA, PCR products were then transferred to fresh DNA LoBind Eppendorf tubes and 30µl of AMPure XP beads added to each sample and incubated with rotation for 5 mins. These beads bind the DNA and, when placed on a DynaMag™-2 Magnet (Fisher Scientific, UK), allow the DNA to be washed while bound. With the samples on the magnet, 200µl of freshly prepared 70% ethanol was run down the side of the tube to wash the beads before being removed. This step was repeated before eluting the beads in 10µl of 10mM Tris-HCl pH 8.0 with 50mM NaCl warmed to 37°C and incubated at room temperature for 2 mins. The samples were then placed back on the magnet and the clear eluate recovered.

The DNA concentration of each sample was measured with a Qubit® 4 fluorometer (Fisher Scientific, UK) using the broad range sensitivity kit, following manufacturer's instructions. Barcoded libraries were then pooled in a 1:1 ratio to a total of 50-100ng in 10mM Tris-HCl pH 8.0 with 50mM NaCl solution. Finally, 1µl RAP was added, incubated at room temperature for 5 mins and the prepared library stored at -20°C for sequencing.

4.2.6 Priming and loading the MinION flow cell.

Before sequencing, the flow cell was primed, following manufacturer's instructions. The flow cell priming mix was prepared by adding 30µl flush tether directly to a tube of flush buffer and mixing thoroughly. Then, 800µl of priming mix was added through the flow cell priming port and incubated for 5 mins. The sequencing library was then prepared with 34µl Sequencing Buffer, 25.5µl Loading Beads, mixed immediately before use, 4.5µl Nuclease-free water and 11 µl DNA library. The SpotON port was then opened and 200µl priming mix added through the priming port to complete priming the flow cell. The library was then loaded in a dropwise fashion in to the SpotON port. Both ports were then closed, and the sequencing run started. A graphical illustration of the ONT MinION® sequencing preparation can be visualised in Figure 4.2.

4.2.7 Statistical analysis.

Microbiome figures were created using the online platform, MicrobiomeAnalyst (Chong et al., 2020) and GraphPad Prism (Version 9, La Jolla, California, USA). All other figures and analyses were performed in GraphPad Prism.

4.3 Results

Key organisms that secrete protective metabolites important to preventing overgrowth of *Candida* in the vagina remain largely understudied. Identifying bacteria which possess mechanisms to maintain vaginal health may lead to improved treatment options for VVC. To observe bacterial taxa present in healthy women and those with RVVC, 16S rRNA sequencing was performed on DNA extracted from high-vaginal swab samples (n = 100). In order to ensure accurate bacterial taxa identification to species-level, two analyses were performed on Illumina sequencing data.

4.3.1 Assessing OTU and ASV analysis pipelines at family and genus-level

The first analysis implemented on Illumina sequencing data using closed reference operational taxonomic unit (OTU) clustering allowing for identification of bacterial taxa present in swab samples to family and genus-level only (Figure 4.3 and 4.4). This analysis observed slightly greater bacterial diversity at both family and genus-level in samples from healthy patients compared with those suffering from RVVC (Figure 4.3A and 4.4A). Further comparison of healthy and diseased samples by principal coordinates analysis (PCoA) could not identify distinct clusters of bacterial taxa at either family or genus-level using this analysis with high levels of overlap between groups (Figure 4.3B and 4.4B).

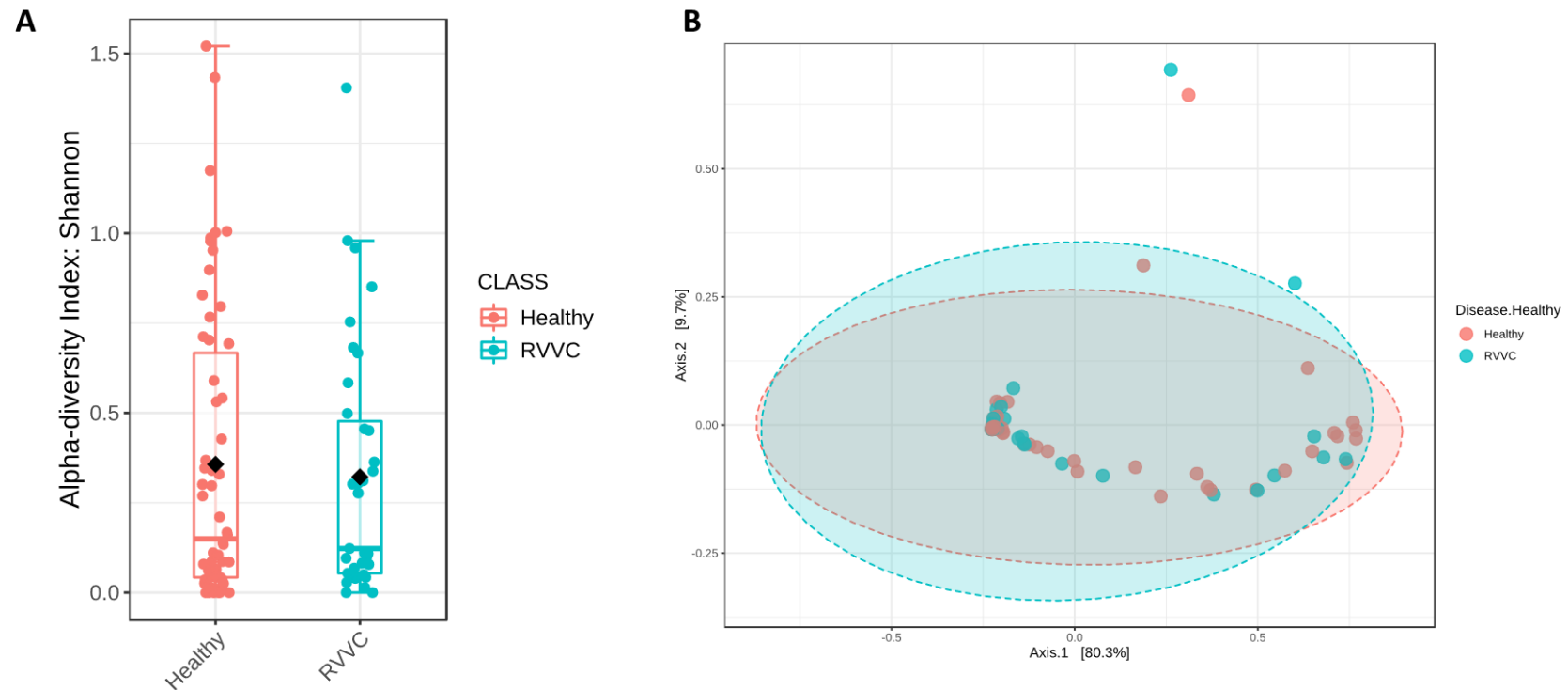


Figure 4.3: Analysis of diversity and Principal Coordinates Analysis of the vaginal microbiome at family-level by Illumina sequencing via closed reference OTU clustering.

DNA extracted from swab samples was used for 16S rRNA Illumina sequencing (n=100). Bacterial diversity in health and RVVC was measured by Shannon index (A). PCoA with Bray-Curtis index to assess variances between health and RVVC (B).

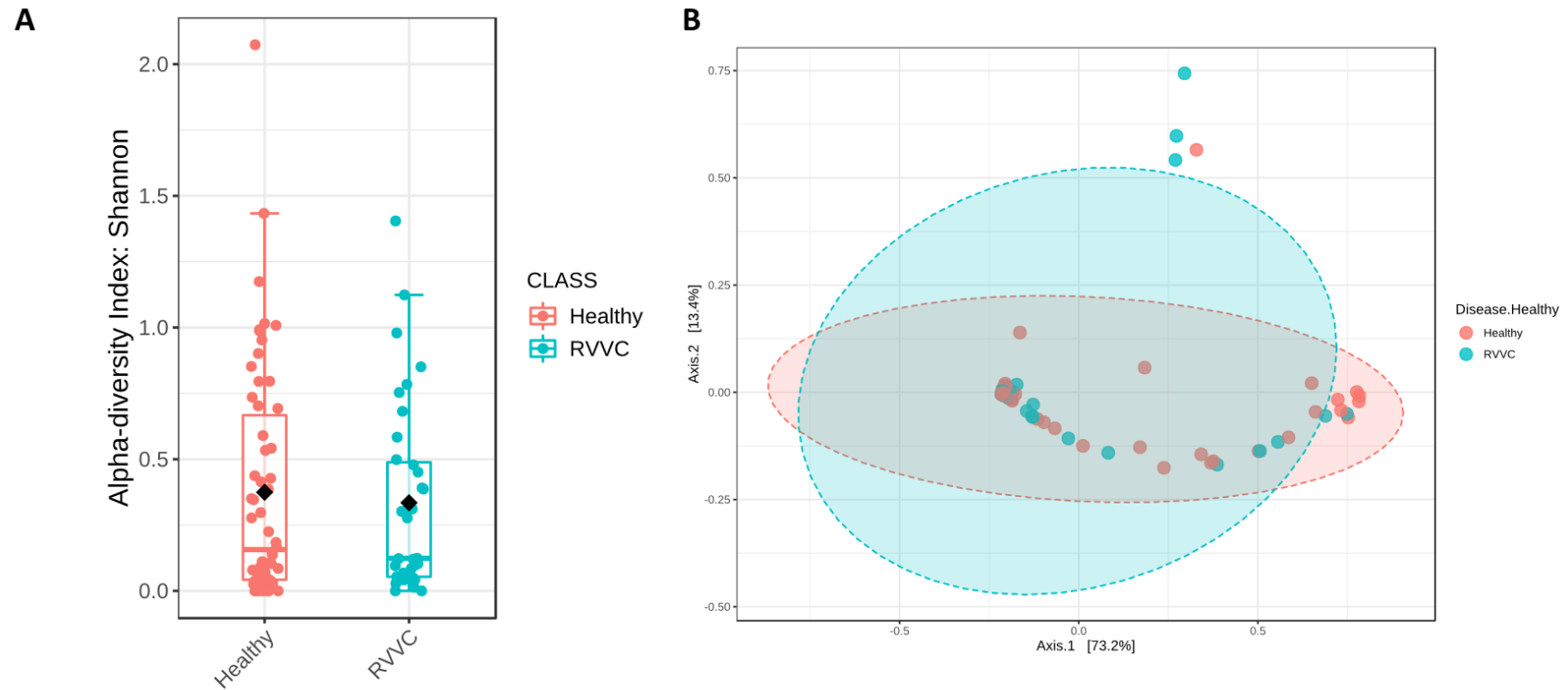


Figure 4.4: Analysis of diversity and Principal Coordinates Analysis of the vaginal microbiome at genus-level by Illumina sequencing via closed reference OTU clustering.

DNA extracted from swab samples was used for 16S rRNA Illumina sequencing (n=100). Bacterial diversity in health and RVVC was measured by Shannon index (A). PCoA with Bray-Curtis index to assess variances between health and RVVC (B).

To investigate differences between the microbial composition during health and RVVC, microbial populations were next identified to family and genus-level using this analysis (Figure 4.5). At family-level, a *Lactobacillaceae*-dominated environment was found in samples from both healthy and diseased patients accounting for 73% and 76% of the population, respectively (Figure 4.5A). Other bacteria identified included predominantly members of the *Bifidobacteriaceae*, *Atopobiaceae* and *Prevotellaceae* families. Few differences in taxa abundances were observed between health and RVVC at this level. At genus-level, *Lactobacillus* was found to govern the microbiome of both healthy and diseased patients accounting for >70% of the total population (Figure 4.5B). Other prevalent genera included *Gardnerella*, *Atopobium* and *Prevotella*. Similarly to family-level, few notable differences in taxa abundance present in health and RVVC were found at genus-level. However, abundances of *Atopobium* were found to be slightly higher in healthy patients and *Bifidobacterium* slightly lower.

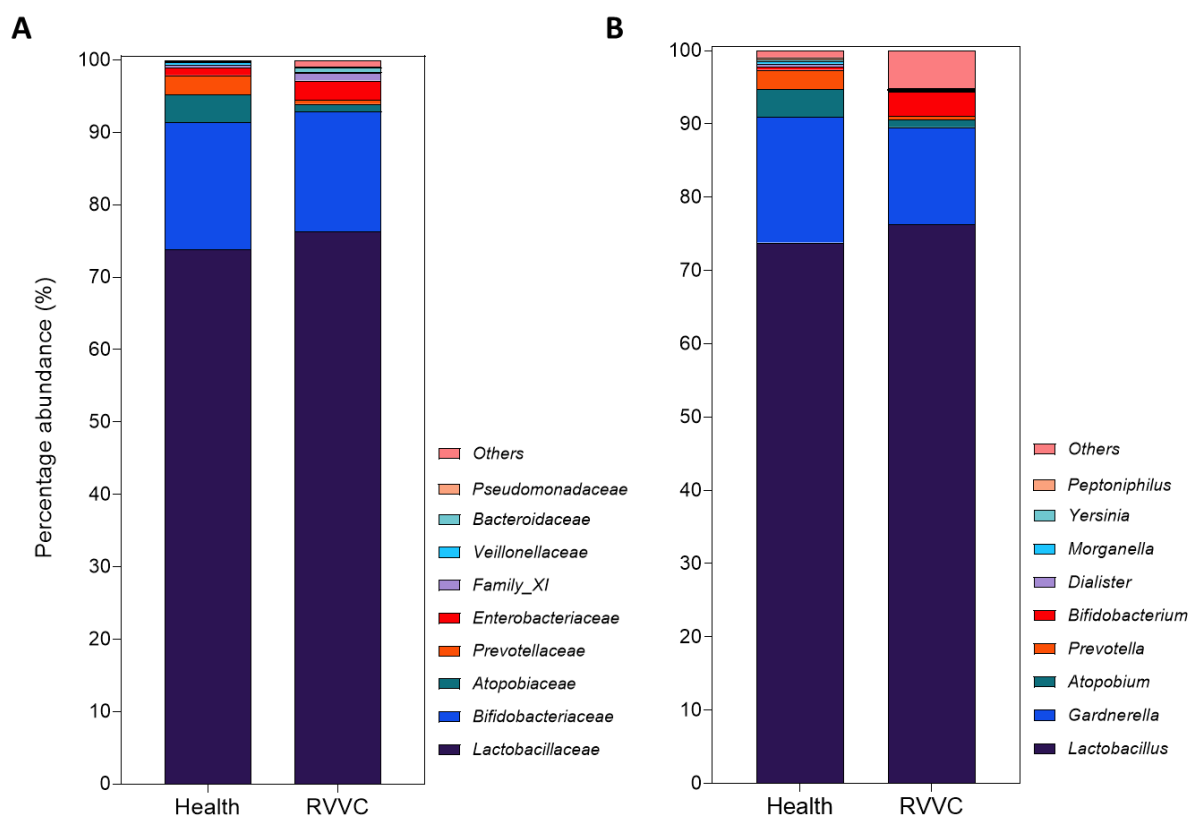


Figure 4.5: Composition of bacterial taxa present in health and RVVC at family and genus-level by Illumina sequencing via closed reference OTU clustering. Bacterial taxa identification to family (A) and genus-level (B) with percentage abundances of microbial populations present in health and RVVC.

The second analysis of Illumina sequencing data utilised inferred amplicon sequence variants (ASVs) to allow for confirmation of family and genus-level taxa as well as allowing for observation of species-level taxa present. This analysis observed slightly greater diversity in microbial populations at both family and genus-level in healthy patients compared with diseased (Figure 4.6A and 4.7A). Analysis by PCoA to identify distinct clusters of bacterial taxa at family and genus-level found overlap of groups with no clear distinction between health and RVVC (Fig 4.6B and 4.7B).

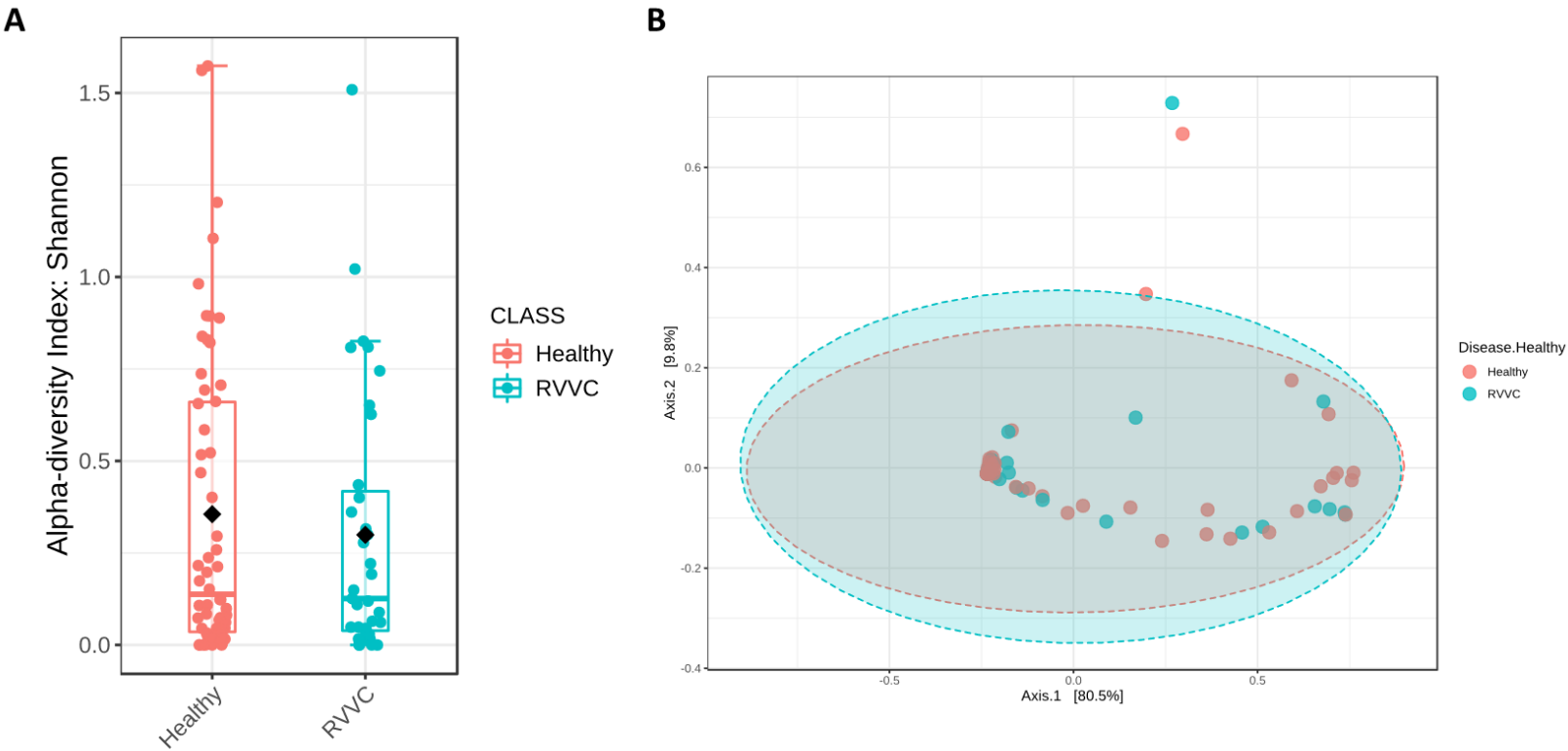


Figure 4.6: Analysis of diversity and Principal Coordinates Analysis of the vaginal microbiome at family-level by Illumina sequencing via inferred amplicon sequence variants (ASV).

Bacterial diversity in health and RVVC measured by Shannon index (A) and PCoA with Bray-Curtis index to assess variances between health and RVVC (B).

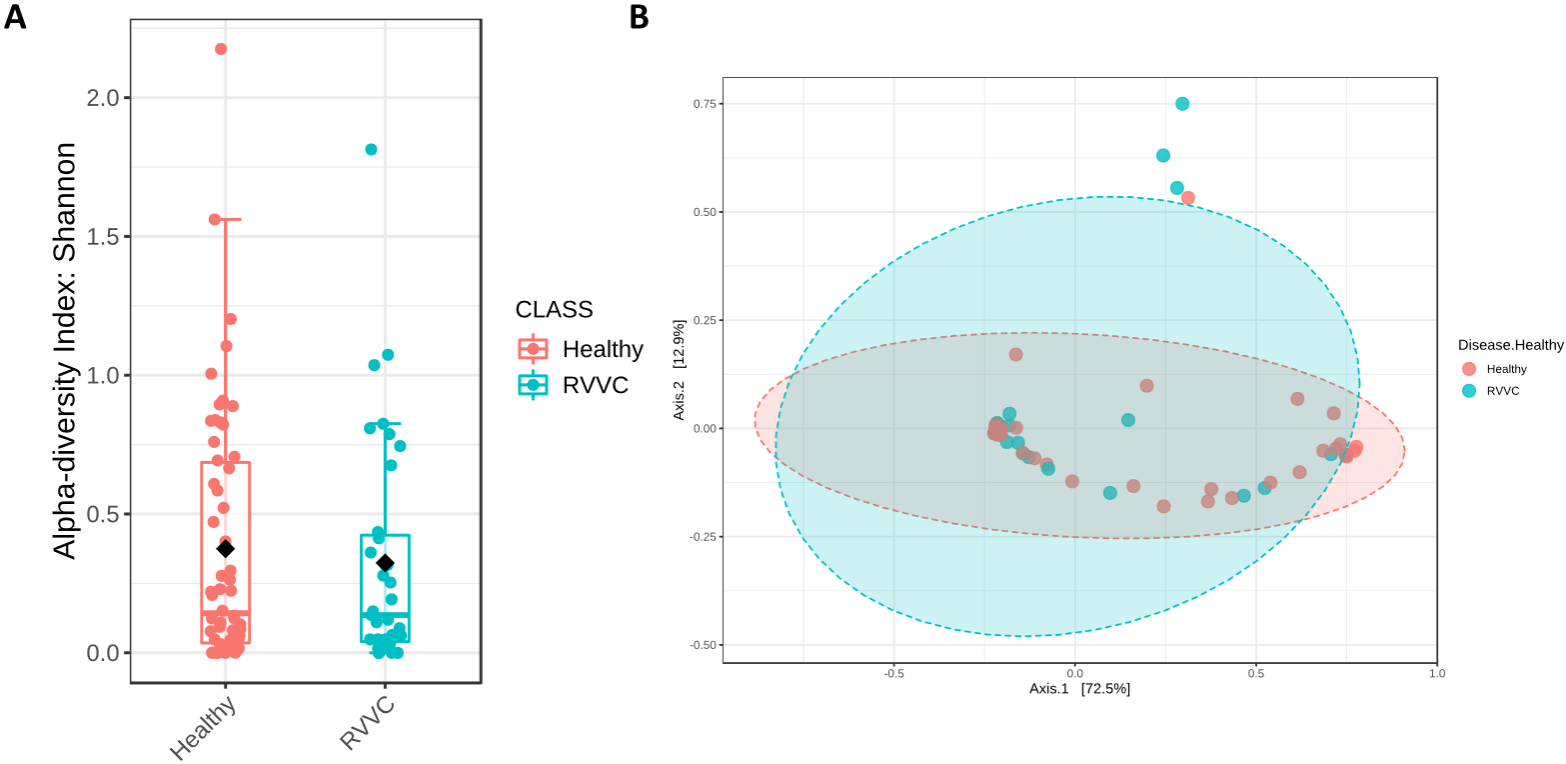


Figure 4.7: Analysis of diversity and Principal Coordinates Analysis of the vaginal microbiome at genus-level by Illumina sequencing via inferred amplicon sequence variants (ASV)

Bacterial diversity in health and RVVC measured by Shannon index (A) and PCoA with Bray-Curtis index to assess variances between health and RVVC (B).

Observation of the bacterial taxa identified to family and genus-level using this analysis found few differences between health and RVVC (Figure 4.8). Again, a *Lactobacillaceae*-dominated environment was seen in both healthy and RVVC samples accounting for up to 74% of the microbiome. Various levels of vaginal anaerobes were also found including those from the *Bifidobacteriaceae*, *Atopobiaceae* and *Prevotellaceae* families (Figure 4.8A). At genus-level, *Lactobacillus* was found to be the dominant genera in both cohorts with no difference in abundance (Figure 4.8B). Both analyses found slightly higher levels of *Gardnerella* and *Atopobium* present in healthy samples.

Overall, as expected at family and genus-level, this analysis showed similar results for diversity and taxa abundance to the previous analysis using OTU clustering. This is confirmatory of accurate bacterial taxa identification at these levels using both analyses. Crucially, this second analysis allowed for species-level identification of microbial populations present during health and RVVC.

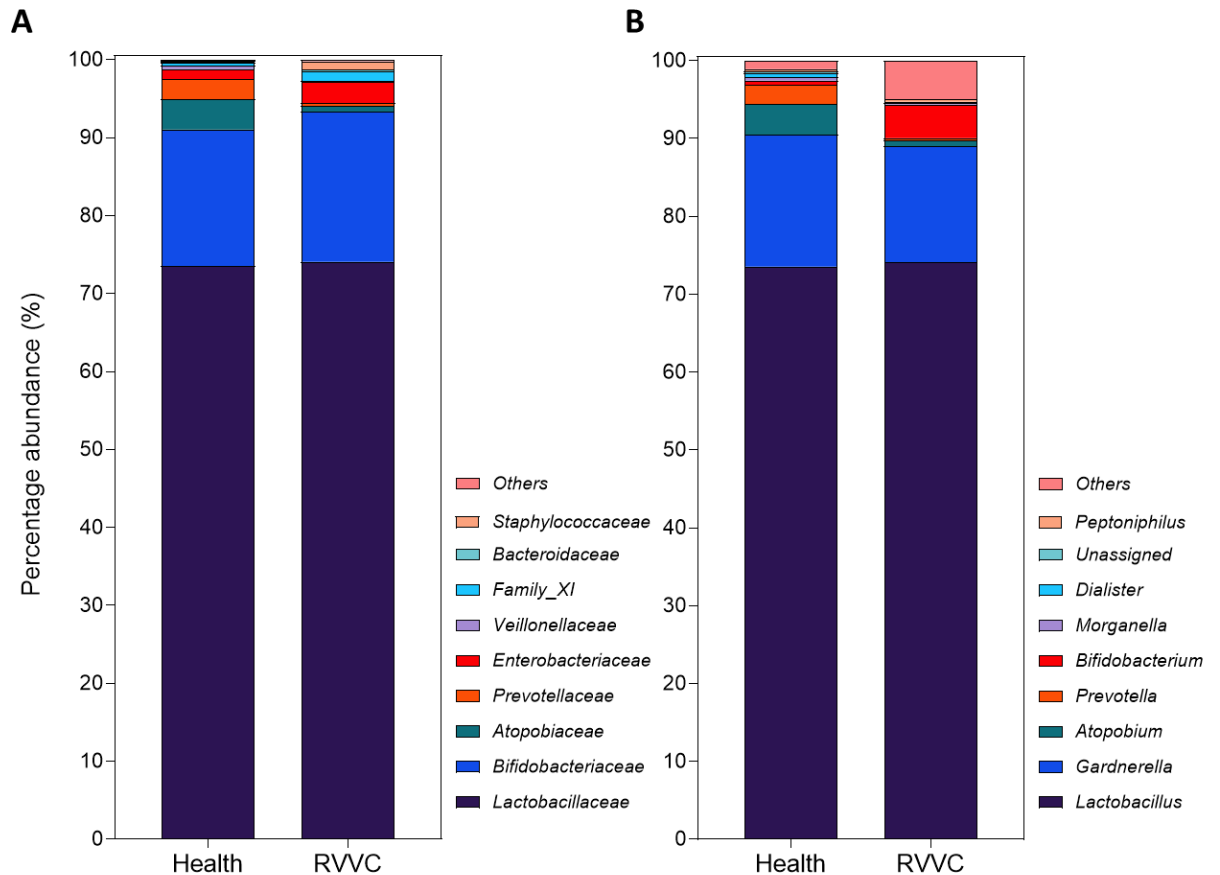


Figure 4.8: Composition of bacterial taxa present in health and RVVC at family and genus-level by Illumina sequencing via inferred ASVs. Bacterial taxa identification to family (A) and genus-level (B) with percentage abundances of microbial populations present in health and RVVC.

4.3.2 Observing bacterial taxa present in health and RVVC at species-level

Species-level identification of the microbial populations present between health and RVVC was imperative for this study to identify potential interkingdom interactions with *Candida* which could influence RVVC pathogenesis. Initial observation of species diversity using the Shannon diversity index found no significant differences between health and disease (Figure 4.9A). Further analysis by PCoA showed clustering of healthy and diseased samples with no clear definition of each cohort (Figure 4.9B).

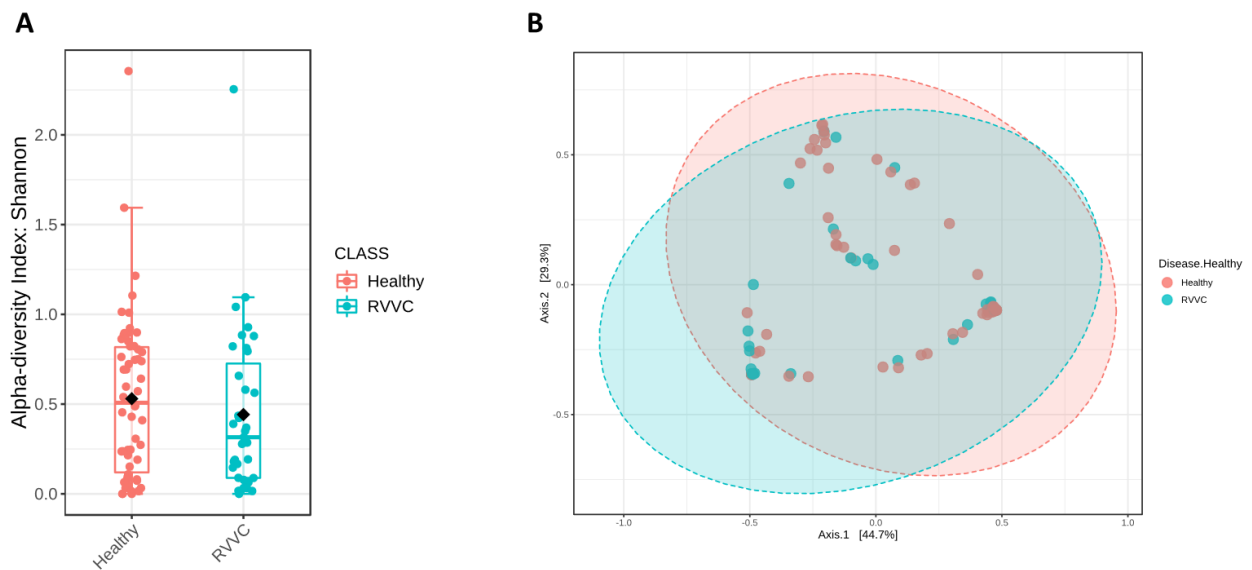


Figure 4.9: Diversity and Principal Coordinates Analysis of vaginal microbiome at species-level. Bacterial diversity measured by Shannon index (A) and PCoA with Bray-Curtis index to assess variances between health and RVVC at species-level.

Analysis of the species-level composition of the microbiome of healthy and RVVC patients revealed subtle key differences in bacterial taxa, particularly amongst *Lactobacillus* species (Figure 4.10). One limitation of this analysis was the inability of the data to accurately distinguish between certain *Lactobacillus* species. For this reason, some species are grouped as one or more possible species. The group labelled “*L. spp. Incl. L. crispatus*” comprised the following species; *L. crispatus*, *L. acidophilus*, *L. casei* and *L. gasseri*. The most notable difference identified was the reduction in levels of specific *Lactobacillus* species including *L. jensenii* and, to a greater extent, *L. crispatus* which fell from 44% in health to 30% in RVVC (Figure 4.10A and 4.10B). These are *Lactobacillus* species commonly associated with a homeostatic, healthy vaginal flora and are thought to reduce risk of dysbiosis through secreted metabolites such as H_2O_2 . Interestingly, this reduction was coupled with an increase in *L. iners* from just 19% in health to 40% in RVVC. Unlike the other species, *L. iners* does not produce H_2O_2 and its presence is becoming recognised as a marker of dysbiosis in the vaginal microbiome. Further, when predicted using a random forest plot, abundance of *L. iners* was found to be the most important feature in distinguishing between the microbiome of health and RVVC (Figure 4.10C). A summary of the bacterial taxa identified to family, genus and species-level and their relative abundances is shown in Table 4.1.

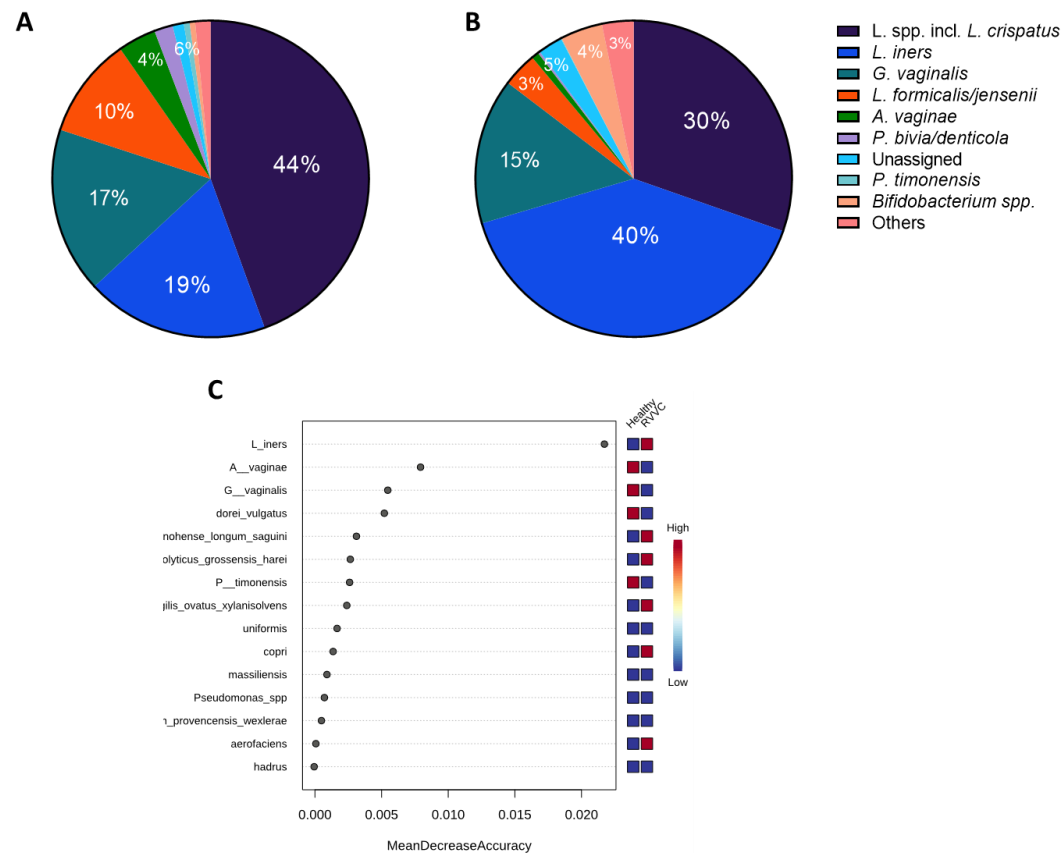


Figure 4.10: Hydrogen peroxide-producing lactobacilli strains are reduced during RVVC resulting in an *L. iners*-dominated microbiome. Species-level identification of bacterial taxa present in health (A) and RVVC (B). Random forest plot showing the most distinct species-level taxa present between health and RVVC (C).

Table 4.1: Percentage relative abundance of most prevalent bacterial taxa at family, genus and species-level between health and RVVC.

Level	Bacterial taxa	Relative abundance (%)		Adjusted P value
		Health	RVVC	
Family	<i>Atopobiaceae</i>	3.900	0.748	0.328
	<i>Prevotellaceae</i>	2.533	0.384	0.565
	<i>Veillonellaceae</i>	0.467	0.052	0.657
	<i>Staphylococcaceae</i>	0.082	1.074	0.657
	<i>Enterobacteriaceae</i>	1.320	2.766	0.675
	Family_XI	0.357	1.191	0.675
	<i>Bacteroidaceae</i>	0.165	0.247	0.675
	<i>Bifidobacteriaceae</i>	17.481	19.245	0.849
	<i>Lactobacillaceae</i>	73.541	74.064	0.948
Genus	<i>Atopobium</i>	3.900	0.748	0.503
	<i>Prevotella</i>	2.494	0.286	0.608
	<i>Bifidobacterium</i>	0.562	4.322	0.608
	<i>Dialister</i>	0.467	0.052	0.608
	<i>Morganella</i>	0.471	0.182	0.608
	Unassigned	0.275	0.150	0.676
	<i>Gardnerella</i>	16.919	14.924	0.843
	<i>Peptoniphilus</i>	0.263	0.241	0.948
	<i>Lactobacillus</i>	73.541	74.064	0.948
Species	<i>L. iners</i>	18.655	40.065	0.311
	<i>A. vaginae</i>	3.900	0.748	0.311
	<i>P. timonensis</i>	0.601	0.065	0.533
	<i>L. formicalis/jensenii</i>	10.302	3.547	0.533
	<i>L. spp. Incl. L. crispatus</i>	44.454	30.374	0.533
	<i>Bifidobacterium spp.</i>	0.562	4.322	0.533
	<i>P. bivia/denticola</i>	1.893	0.221	0.533
	Unassigned	1.143	2.499	0.567
	<i>G__vaginalis</i>	16.919	14.924	0.832

To ensure identity specificity of bacterial identification at species-level, the microbiome of a subset of samples was analysed using the Oxford Nanopore Technologies MinION sequencer (Tyler et al., 2018). For this purpose, ten samples with the highest *Lactobacillus* counts were selected from each cohort (n = 20). This sequencer, unlike Illumina sequencing platforms, sequences the full variable region of the 16S rRNA gene allowing for high-specificity, accurate identification to species-level. Sequencing this subset of samples allowed for direct comparison of Illumina and Nanopore sequencing platforms as well as ensuring accurate species-level identification had been achieved before further analysis. Comparing the two sequencing techniques at genus-level revealed some differences in composition (Figure 4.11). Although both analyses found a *Lactobacillus*-dominated environment in both health and RVVC, sequencing

with Nanopore was able to identify higher numbers of other genera. Further, where Illumina showed almost identical levels of *Lactobacillus* in health and RVVC, Nanopore sequencing observed an increase in *Lactobacillus* levels from 85% to 96% in RVVC. Other genera identified by Nanopore sequencing included *Streptococcus*, *Haemophilus* and *Campylobacter*.

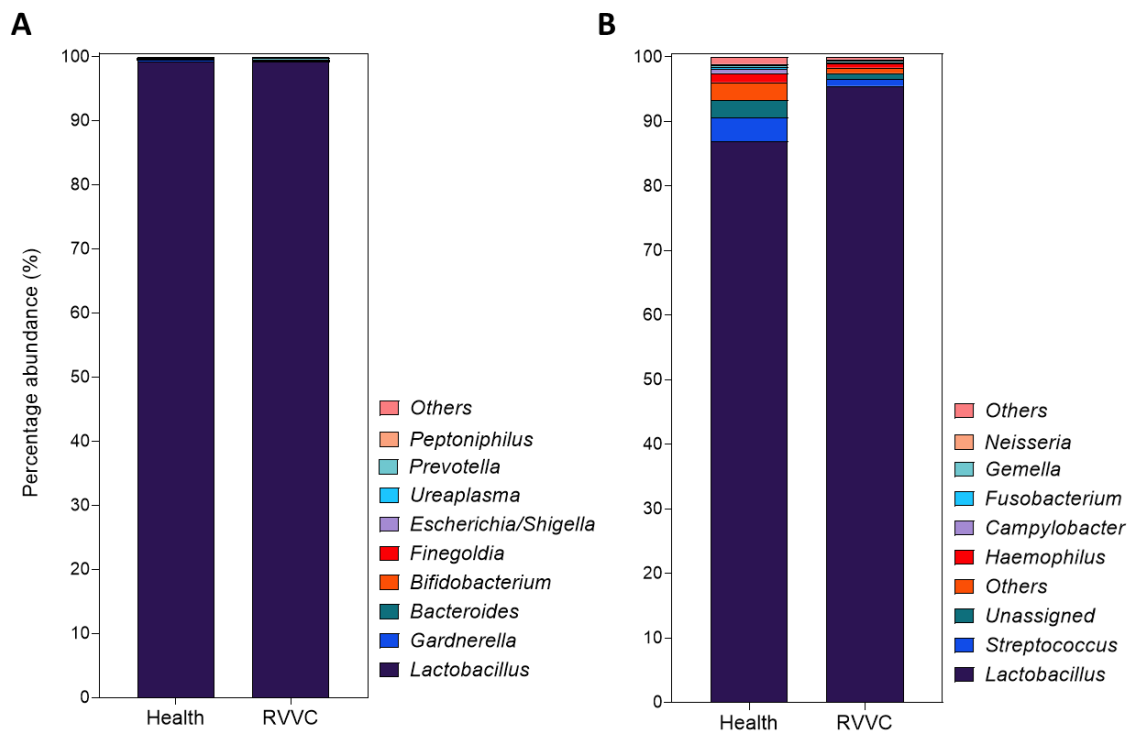


Figure 4.11: Specificity of genus-level identification is comparable across Illumina and Nanopore sequencing platforms. Bacterial taxonomy identification by Illumina to genus-level (A) compared with Nanopore MinION identification to genus-level (B).

At species-level, further differences can be seen between sequencing platforms (Figure 4.12). Where Illumina sequencing found 47% of the sample subset to be comprised of *Lactobacillus* species, including *L. crispatus* in both health and RVVC, Nanopore identified a slight difference of 4% between the cohorts. Further, Nanopore sequencing allowed for more accurate distinction between genetically similar *Lactobacillus* species, meaning certain species did not require grouping for this analysis. This is seen in Illumina data where much higher levels of *L. iners* are found in both cohorts, however Nanopore has identified some of these microbes as *L. amylovorus*, *L. kitasatonis* and *L. jensenii*. Overall, although there is some fluctuation in abundances, this analysis confirmed that accurate identification of *Lactobacillus* species by Illumina sequencing.

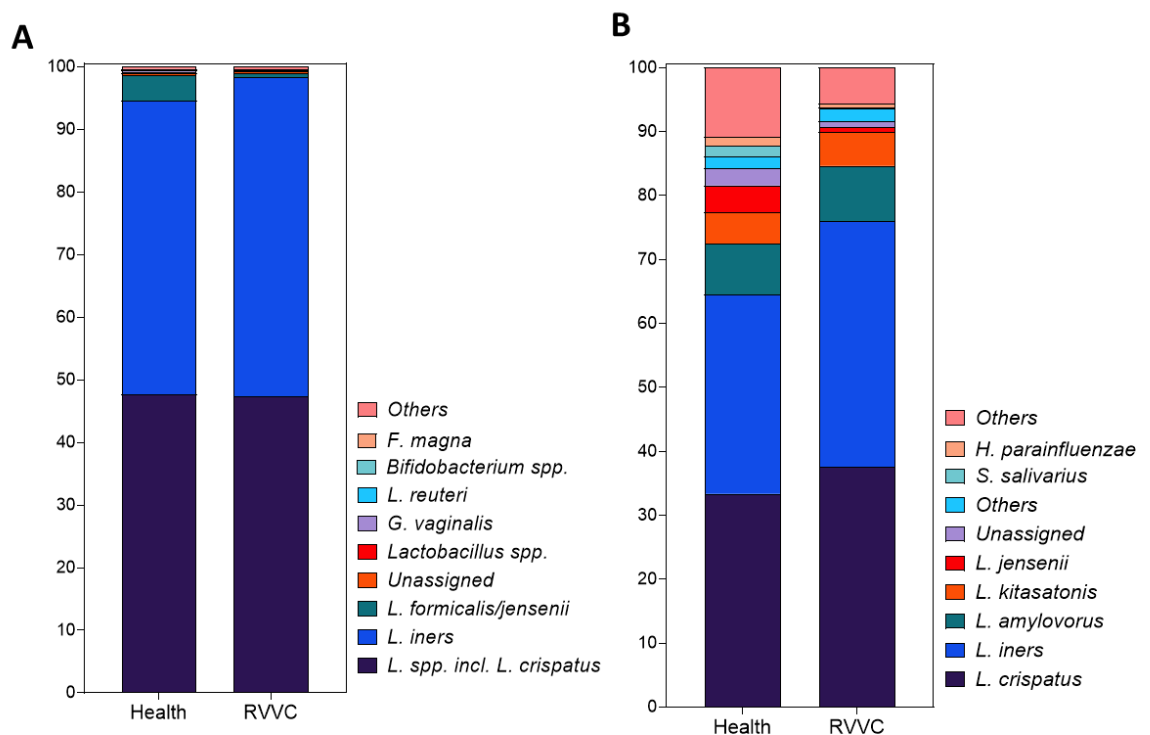


Figure 4.12: Specificity of species-level identification is comparable across Illumina and Nanopore sequencing platforms. Bacterial taxonomy identification by Illumina to species-level (A) compared with Nanopore MinION identification to species-level (B).

4.3.3 *The influence of lifestyle and treatment on the microbiome*

To investigate the influence of bacterial taxa further, microbial populations were next observed with respect to patient metadata. At genus-level, patients with culturable *Candida* compared with those who were culture-negative showed a reduction in composition of *Lactobacillus* from 76% to 68% (Figure 4.13A). Further, patients with no culturable *Candida* have slightly higher levels of *Atopobium*. These observations for patients with culturable *Candida* are similar to those seen in diseased patients. At species-level, a reduction in *Lactobacillus* species including *L. crispatus* from 44% to 29%, coupled with an increase in *L. iners* from 23% to 35% was observed (Figure 4.14A). Additionally, levels of *G. vaginalis* were 3% lower in culture-negative patients with higher quantities of *L. formicalis/jensenii* and *A. vaginae*. This observation is again similar to that of the RVVC profile seen when comparing health and disease. Further, random forest plots were constructed to predict the most important feature in distinguishing between the two cohorts. This analysis found high levels of *Gardnerella*, followed by low level of *Lactobacillus*, to be the most important features in identifying patients with culturable *Candida* (Figure 4.13B). The most important features to identify *Candida*-positive samples at species-level were predicted to be high levels of *L. iners* and low levels of *Lactobacillus* species including *L. crispatus* and *L. formicalis/jensenii* (Figure 4.14B).

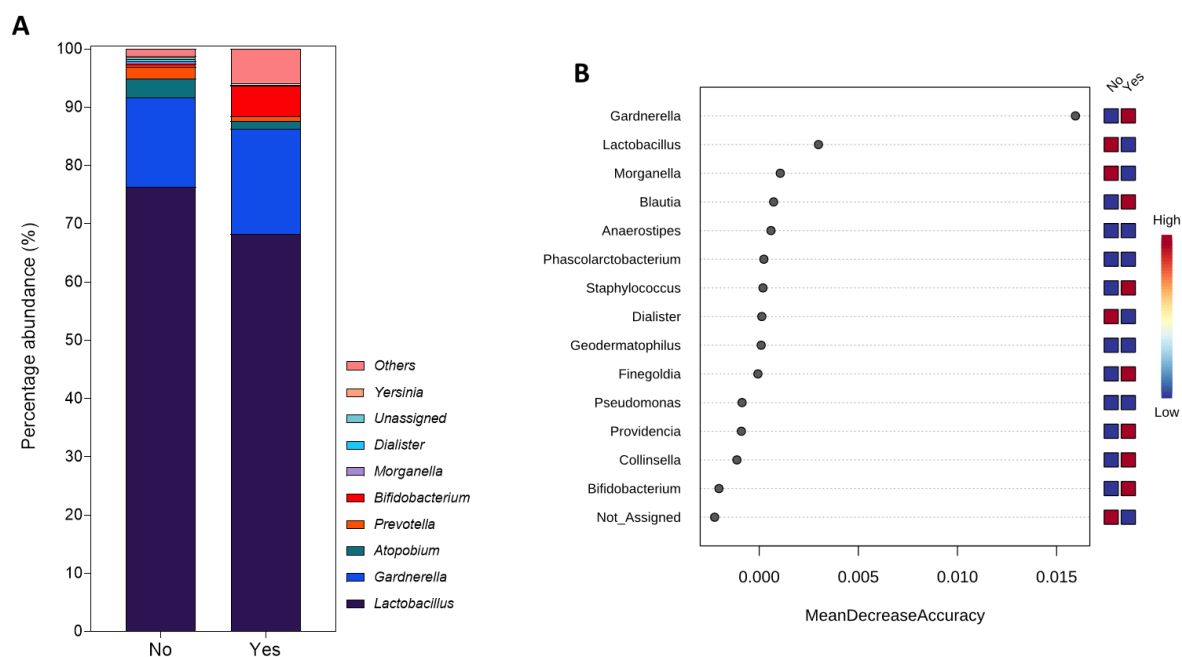


Figure 4.13: Genus-level taxa abundance relative to presence/absence of culturable *Candida*. Bacterial taxa present based on presence/absence of culturable *Candida* at genus-level (A). Random forest plots showing bacterial taxa ranked by their contribution to each classification (B).

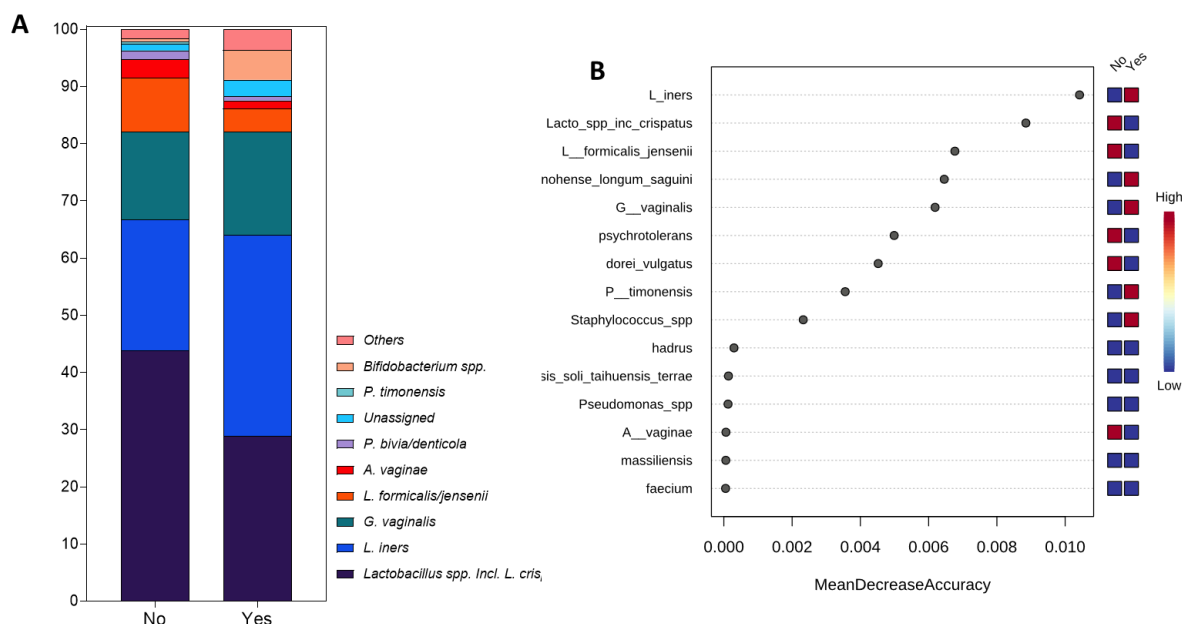


Figure 4.14: Species-level taxa abundance relative to presence/absence of culturable *Candida*. Bacterial taxa present based on presence/absence of culturable *Candida* at species-level (A). Random forest plots showing bacterial taxa ranked by their contribution to each classification (B).

Next, the bacterial composition of the samples was assessed with respect to the form of contraception used by patients at the time of sampling. At genus-level, few differences in bacterial composition were observed between contraception types (Figure 4.15A). In all cases, *Lactobacillus* accounted for around 73% of the composition, followed by *Gardnerella* at 11-17%. At species-level, some substantial differences can be seen between health and different contraception types (Figure 4.16A). Most notably is the similarity observed in the composition of healthy patients and those using hormonal contraception in contrast with patients using a contraceptive device or no contraception. A sharp decrease in levels of *Lactobacillus* species including *L. crispatus* is observed from 44% in health to 15% and 20% in patients using a contraceptive device or no contraception, respectively. This trend is absent in patients using hormonal contraceptives where levels increase slightly to 48%. This decrease is coupled with an increase of *L. iners* from 18% in health to 57% and 44% in patients using device or no contraception, respectively. Further, levels of *A. vaginae* and *L. formicilis/jensenii* were reduced from 4% and 10%, respectively, in health to <2% in patients using contraceptives. At genus-level, *Lactobacillus*, *Gardnerella* and *Prevotella* were predicted to be the most important features to distinguish between different forms of contraception (Figure 4.15B). The most important feature identified at species-level was predicted to be *A. vaginae* (Figure 4.16B). It is predicted that this organism is higher in healthy patients and those using hormonal contraceptives, and lower in patients using either no contraception or contraceptive devices. Additionally, a contrasting pattern is shown for *L. iners*, which is predicted to be highest in patients using contraceptive devices.

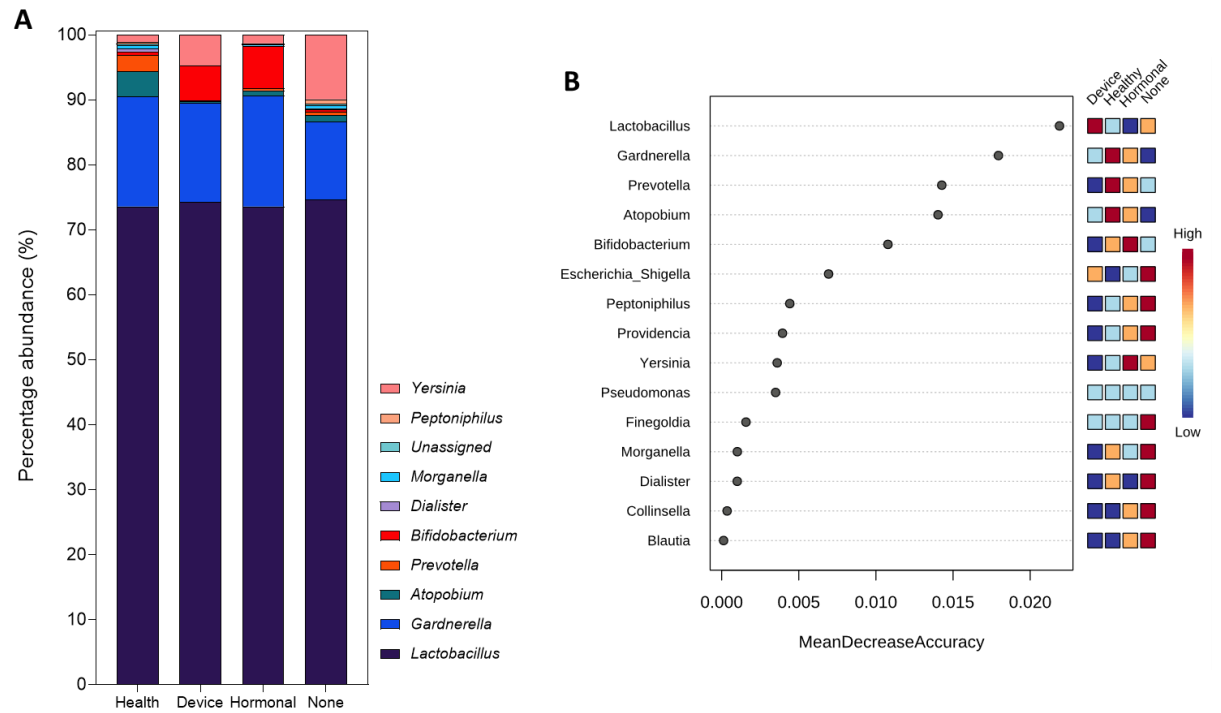


Figure 4.15: Genus-level taxa abundance relative to contraception used. Bacterial taxa present based on the contraception used by patients at genus-level (A). Random forest plots showing bacterial taxa ranked by their contribution to each classification (B).

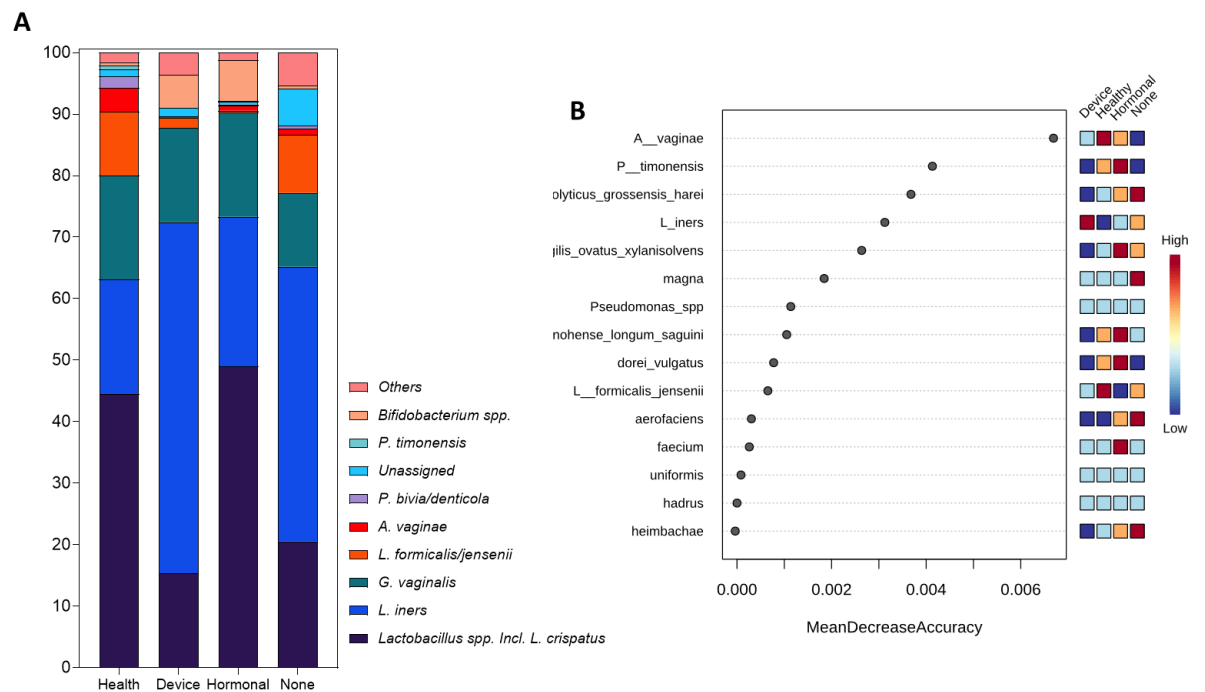


Figure 4.16: Species-level taxa abundance relative to contraception used. Bacterial taxa present based on the contraception used by patients at species-level (A). Random forest plots showing bacterial taxa ranked by their contribution to each classification (B).

The microbiome of patients was then assessed with respect to how recently patients had received antifungal intervention to treat RVVC. Few changes were noted at genus-level between health and treatment times (Figure 4.17A). However, *Lactobacillus* was slightly increased to 76% in those treated more recently and reduced to 69% in patients treated >1 month before sampling. At species-level, a gradual reduction in *Lactobacillus* species including *L. crispatus* can be seen from 44% in health to 34% and 23% in patients treated within a month and over a month before sampling, respectively (Figure 4.18A). Further, those treated more recently had 5% higher levels of *G. vaginalis* and 6% lower levels of *L. formicalis/jensenii*. High levels of *Prevotella* and *Atopobium* were predicted to be the most important feature in defining health with low levels being more closely associated with those treated within one month of sampling (Figure 4.17B). Conversely, *L. iners* was predicted to be higher in patients treated within one month and lower in health (Figure 4.18B). Patients treated over one month before sampling were predicted to have an intermediate profile between health and those treated more recently.

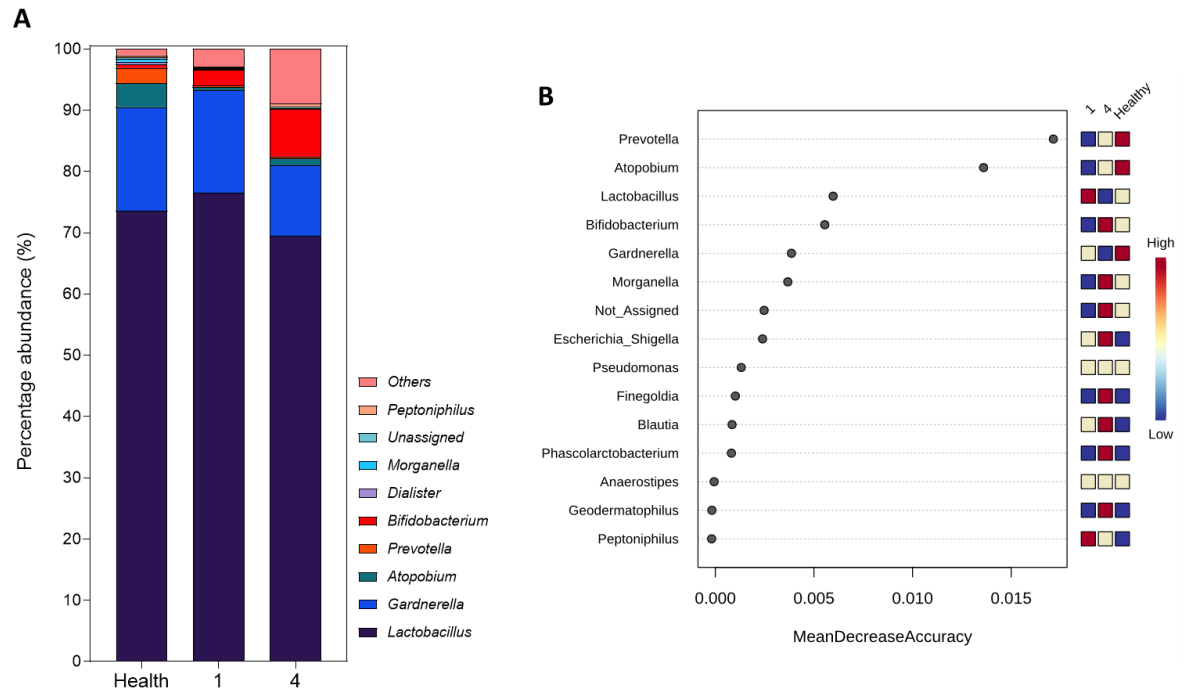


Figure 4.17: Genus-level taxa abundance relative to how recently patients had received antifungal treatment. Bacterial taxa present based on time since antifungal treatment (1 = ≤1 month and 4 = >1 month prior to sampling) at genus-level (A). Random forest plots showing bacterial taxa ranked by their contribution to each classification (B).

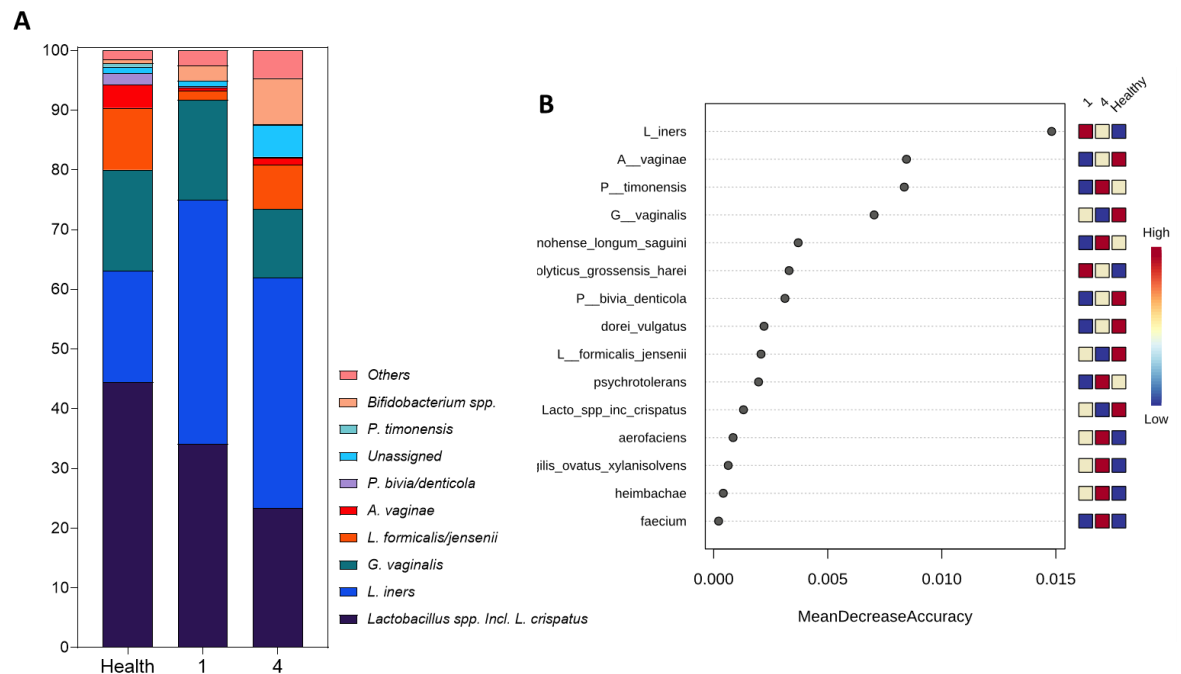


Figure 4.18: Species-level taxa abundance relative to how recently patients had received antifungal treatment. Bacterial taxa present based on time since antifungal treatment (1 = ≤1 month and 4 = >1 month prior to sampling) at species-level (A). Random forest plots showing bacterial taxa ranked by their contribution to each classification (B).

When observed with respect to the length of time patients had suffered from RVVC, an intermediate profile between health and disease was observed in patients with disease for <6 months. An initial increase in *Lactobacillus* was noted from 73% in health to 83% in patients with disease for ≤ 6 months, followed by a decrease to 66% in patients with disease for >6 months (Figure 4.19A). Additionally, levels of *Bifidobacterium* were increased from <1% to 8% in patients with RVVC for >6 months. This intermediate profile was also noted at species-level where levels of *Lactobacillus* including *L. crispatus* are reduced from 44% in health to 39% in patients with disease for <6 months, followed by a sharp decrease to 22% in patient with RVVC for >6 months (Figure 4.20A). This is once again coupled by an increase in *L. iners* composition from 18% in health to 36% and 44% in patients with disease for <6 month and >6 months, respectively. Additionally, levels of *L. formicalis/jensenii* and *A. vaginae* are also reduced over time from 7% in patients with disease for <6 month to <0.5% in patients with RVVC for >6 months. *Lactobacillus* was predicted to be the most important feature in distinguishing between disease profiles with higher levels found in healthy patients and those with disease for <6 months (Figure 4.19B). This was closely followed by *Atopobium*, *Gardnerella* and *Prevotella* which were predicted to be highest in healthy patients and lowest in patients with disease for <6 months. The most important species identified were *L. iners*, where high levels indicate patients with disease for longer, and *L. formicalis/jensenii* where high levels are associated with health (Figure 4.20B).

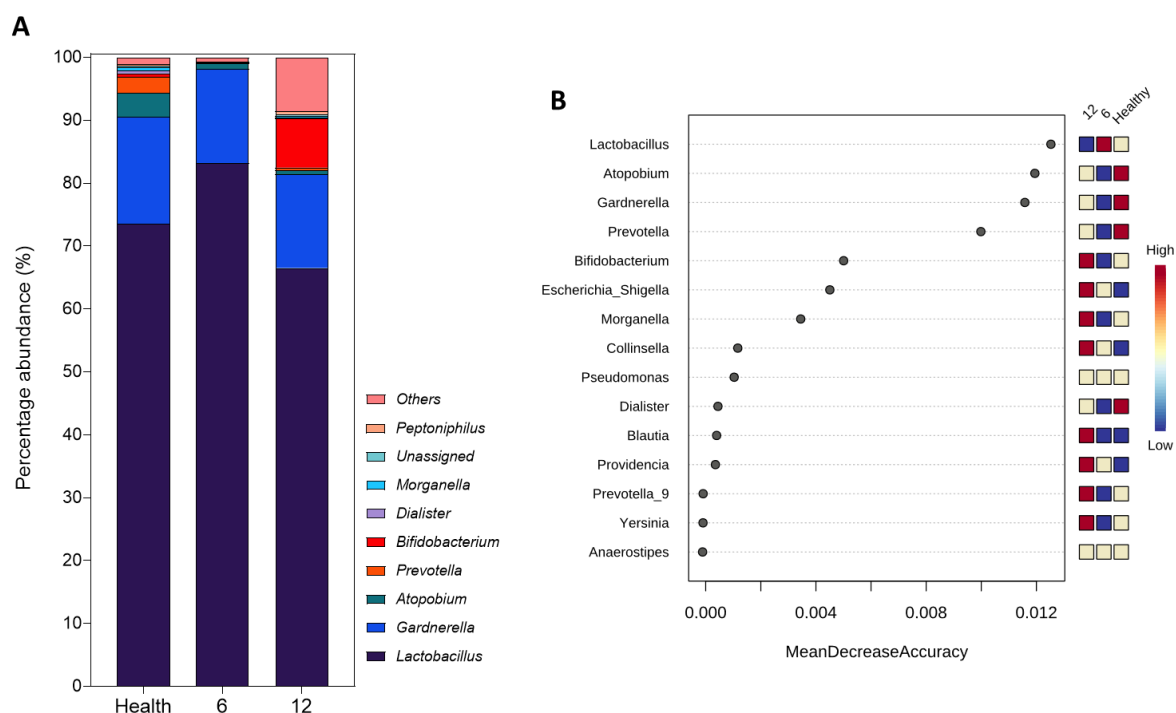


Figure 4.19: Genus-level taxa abundance relative to how long patient had suffered from RVVC.

Bacterial taxa present based on length of time with disease (6 = ≤6 months and 12 = 7-12 months) at genus-level (A). Random forest plots showing bacterial taxa ranked by their contribution to each classification (B).

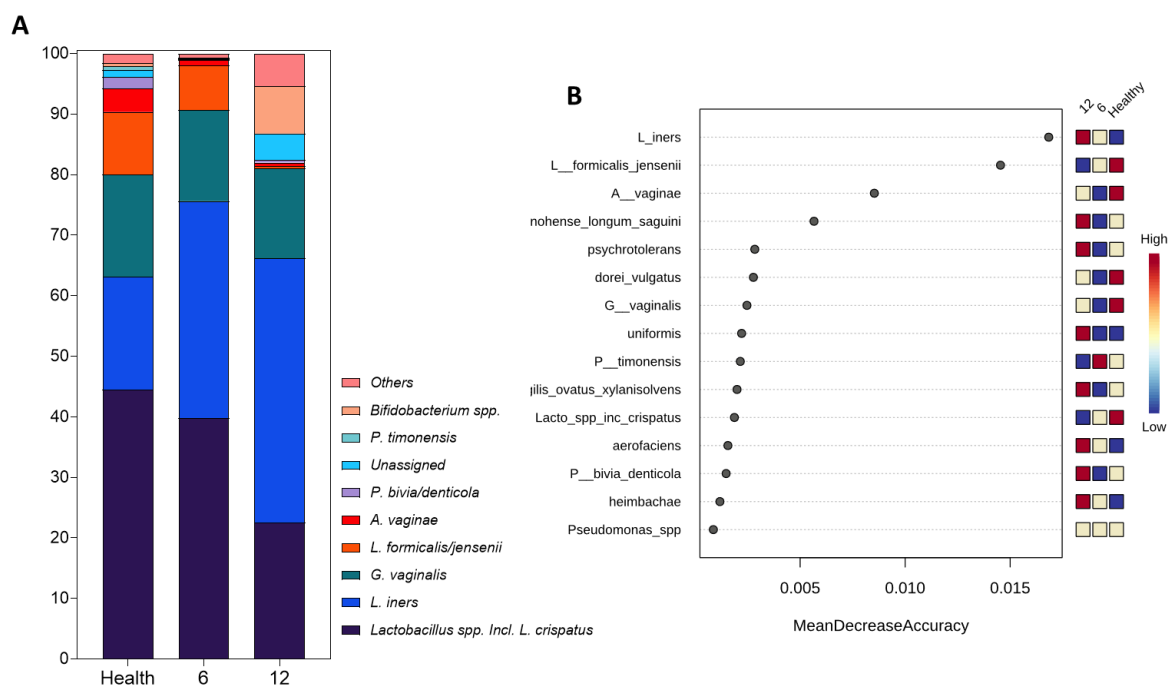


Figure 4.20: Species-level taxa abundance relative to how long patient had suffered from RVVC.

Bacterial taxa present based on length of time with disease (6 = ≤6 months and 12 = 7-12 months) at species-level (A). Random forest plots showing bacterial taxa ranked by their contribution to each classification (B).

Finally, correlation between the vaginal microbiome and *Candida* load (CFE/mL) was observed with respect to patient metadata (Figure 4.21). *Megasphaera*, often associated with vaginal dysbiosis, was found to be significantly positively correlated with increased fungal load ($P < 0.05$). Although not significant, genera such as *Dialister*, *Bacteroides* and *Shuttleworthia* were correlated with higher fungal loads in RVVC. Further, *Lactobacillus* was significantly negatively correlated with *Candida* in health, and to a lesser extent in RVVC ($P < 0.05$). Although none were found to be significant, correlations observed with respect to recent treatment identified slight negative correlations between genera such as *Streptococcus*, *Gardnerella* and *Lactobacillus* with increasing time since last RVVC intervention. Conversely, genera such as *Prevotella*, *Morganella* and *Sneathia* were found to be positively correlated with increasing time since treatments. *Streptococcus*, *Prevotella* and *Clostridium* were found to be positively correlated with increased RVVC episodes. However, genera such as *Shuttleworthia*, *Lactobacillus* and *Atopobium* were found to be negatively correlated with increasing RVVC episodes.

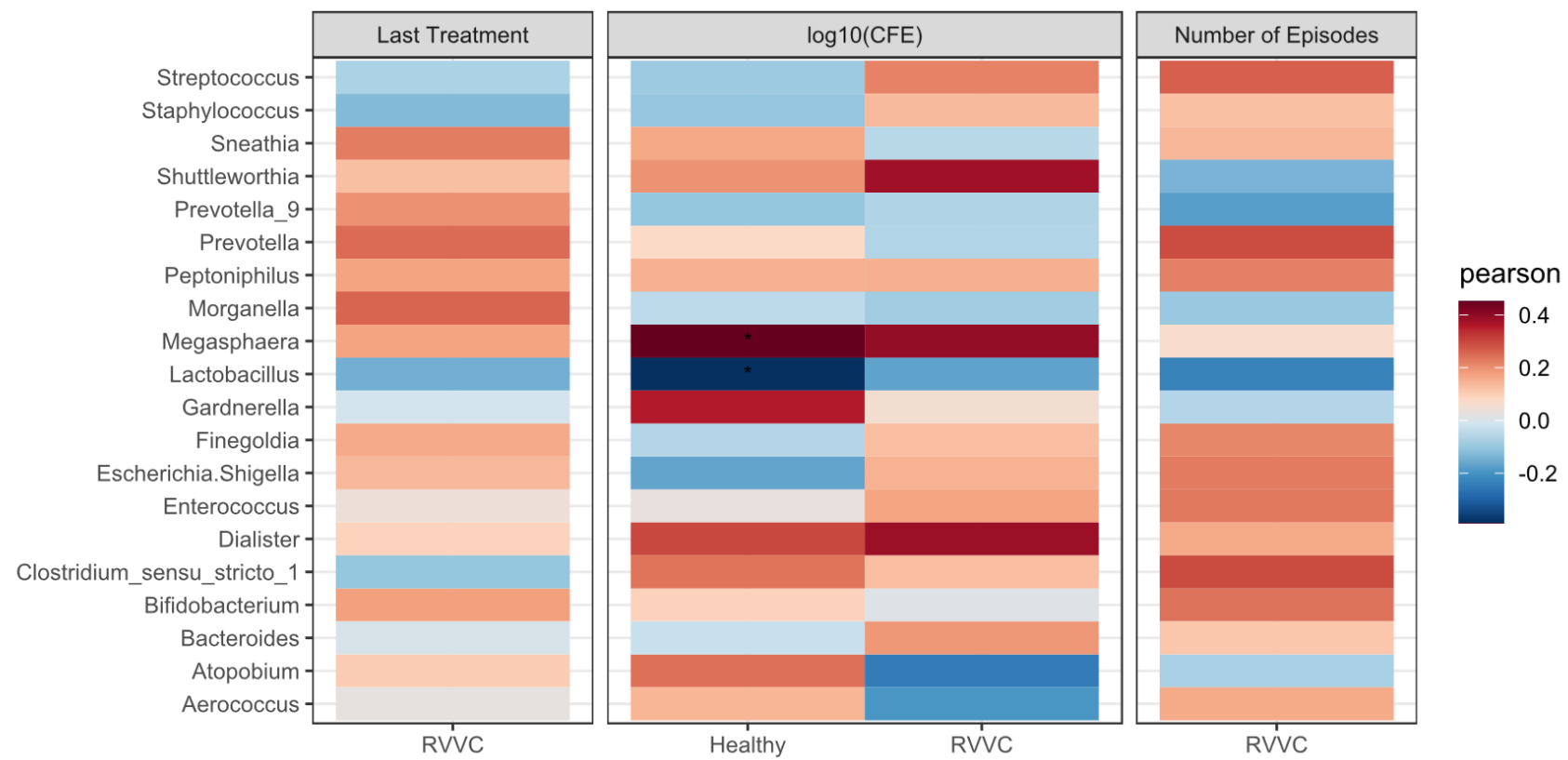


Figure 4.21: Correlation of the microbiome and fungal load with patient metadata.

Heatmap displaying correlations between bacterial genera present and *Candida* load (CFE/mL), recent treatment and number of episodes of RVVC. Significance was measured using Pearson correlation tests (*, $P < 0.05$).

4.4 Discussion

Unlike vaginitis caused by BV, the microbial communities present during VVC have been shown to be similar to those present in health at the family and genus-level (Ceccarani et al., 2019, Ma et al., 2016). For this reason, the potential importance of interkingdom interactions with *Candida* species during VVC has remained largely overlooked. Elucidating organisms which have the potential to prevent or disrupt vaginal overgrowth of *Candida*, as well as those which do not have protective properties, may pave the way to future therapeutic options involving probiotic treatments for women with debilitating RVVC.

Initially, diversity analysis of Illumina sequencing data via closed reference OTU clustering found similarities at both family and genus-level of bacterial taxa present in health and RVVC. This data suggests there were no differences between bacterial richness or evenness between health and RVVC in this patient group. Further, analysis by PCoA with Bray-Curtis index found no significant variations between grouping of bacterial taxa in health and RVVC. These findings were confirmed with a second analysis of Illumina sequencing data via inferred ASVs. Authors have previously reported that biodiversity is augmented in vaginal diseases such as BV and *C. trachomatis* compared with health (Ceccarani et al., 2019, Filardo et al., 2017). However, the diversity profile seen in VVC has been identified to be not significantly different from health or, in some cases, intermediate to the disease profile (Ceccarani et al., 2019, Liu et al., 2013b, Macklaim et al., 2015). When observing the bacterial taxa present in health and RVVC at family and genus-level, a *Lactobacillus*-dominated environment with members of the *Bifidobacteriaceae*, *Atopobiaceae* and *Prevotellaceae* families comprising *Gardnerella*, *Atopobium* and *Prevotella* was found in both cohorts with no significant differences. *Gardnerella*, although primarily associated with biofilm formation in BV, was found to account for ~15% of the microbiome of both healthy and RVVC patients in this study. Although this could be an indicator of disease caused by both BV and VVC, it is more likely that this is due to asymptomatic colonisation and has been identified previously in vaginal microbiome studies (Ceccarani et al., 2019, Liu et al., 2013b). Taxonomic identification and abundances were confirmed in the second analysis using inferred ASVs. Although it is hypothesised that VVC results from a reduction of *Lactobacillus*

due to overgrowth of *Candida* in the vagina, microbiome studies have often found there to be no significant reduction in *Lactobacillus* in diseased women (Zhou et al., 2009a, Macklaim et al., 2015, McClelland et al., 2009). As a result, these authors have rejected this hypothesis and disregarded the potential health benefit of probiotic therapies using *Lactobacillus* species. However, this observation does not account for potential fluctuations in abundances of individual *Lactobacillus* species which may provide protection against dysbiosis and onset of vaginitis. This suggests that the functional capacity of the bacterial species found in health and RVVC may play a more crucial role in disease pathology.

Carrying out the second analysis of Illumina sequencing data proved crucial in identifying key *Lactobacillus* species present in health and RVVC. A limitation of the microbiome analysis was the inability to distinguish between some *Lactobacillus* species (including *L. acidophilus*, *L. casei* and *L. gasseri*). For this reason, these species are grouped as '*L. spp*, incl. *L. crispatus*'. Initially, diversity analysis showed similar levels of species richness and consistency between health and RVVC with significant overlap of ellipses when viewed by PCoA. However, although equally diverse, analysis of the species present between the two cohorts revealed important differences, particularly amongst *Lactobacillus* species. The reduction seen in protective H₂O₂-producing species such as *L. crispatus* and *L. jensenii* has been reported previously in patients with disease (Ceccarani et al., 2019). Additionally, inhibitory properties of *L. crispatus* against *C. albicans* have been demonstrated (Wang et al., 2017, Jang et al., 2019). It is thought that this protective characteristic may be, in part, due to the ability of *L. crispatus* to produce both the L- and D-lactic acid isomers (Amabebe and Anumba, 2018). This data also found an elevation in the non-H₂O₂-producing strain *L. iners* in patients with RVVC. Authors have previously reported this increase in *L. iners*, although non-pathogenic in itself, to be an indicator of dysbiosis of the vaginal microbiome (Ceccarani et al., 2019). One explanation for *L. iners*' lack of protection is that out of the four most common vaginal *Lactobacillus* species, *L. iners* is the only species incapable of producing the more protective L-isomer of lactic acid (Witkin and Linhares, 2017, Witkin et al., 2013). Although consistent, the trends identified in this study were not statistically significant. This is likely due to a relatively limiting sample size. It is becoming more widely recognised that although an overall reduction in *Lactobacillus* is not

seen in VVC episodes, important fluctuations in key *Lactobacillus* species have been found. Therefore, it is proposed that *L. crispatus* could contribute to maintenance and restoration of vaginal homeostasis during vaginal disease such as RVVC. To investigate this hypothesis, in depth analyses of the interkingdom interactions between *L. crispatus* and *Candida* species using sequencing technologies, such as those carried out in Chapter 5 of this thesis are required. A deeper understanding of these mechanisms could lead to greatly improved treatment options through *L. crispatus* probiotic therapies.

To remove ambiguity of the *Lactobacillus* species that could not be distinguished by Illumina sequencing, a subset of samples was sequenced using the Oxford Nanopore Technologies MinION sequencing platform to confirm accurate identification. Similar studies have compared both platforms for the analysis of the nasal microbiome as well as environmental communities (Heikema et al., 2020, Nygaard et al., 2020). Our analysis found overall consistency between genus and species-level identification across the two sequencing platforms with some variation in abundances. Despite these fluctuations, both platforms confirm decreased levels of *L. crispatus* and increased levels of *L. iners* in RVVC compared with health. This data could suggest Illumina overestimates the quantity of one bacterial genus when it accounts for significant proportions of the microbiome. This would propose that Nanopore may be a better tool when accurate identification to species-level is required. Although assumptions can be made, it is impossible to determine which analysis was more accurate in this study. Nanopore is capable of a more accurate species identification as it sequences all nine 16S rRNA hypervariable regions compared with only the V4 region utilised by Illumina in this study. However, Nanopore is still a relatively new technology and its accuracy of ~95% is still considerably lower than 99.9% of the Illumina MiSeq platform (Santos et al., 2020). This is mainly a result of longer reads introducing errors through insertions/deletions (Cole et al., 2007). This is likely why species such as *L. amylovorus* and *L. kitasatonis*, which are not commensals of the human vaginal environment, were identified by this method. Further, analysis tools for data produced by Nanopore sequencers are limited meaning one limitation of this study is the potential for misinterpretation of Nanopore sequencing data due to the analysis employed (Santos et al., 2020). When more appropriate tools are developed for this analysis,

future studies directly comparing these platforms using known polymicrobial communities are required to determine variances in identification and abundance at species-level. This technology is still very much evolving and requires optimisation before it can be confidently used for clinical analyses.

To investigate the role of the microbiome in RVVC further, bacterial taxa present were observed with respect to patient metadata collected via questionnaires. Although not all diseased patients had culturable *Candida* (40% negative), and not all healthy patients were culture-negative (15% positive), microbiome analysis found patients with culturable *Candida* to have a similar microbial profile to those with symptomatic disease. A similar trend of increased *L. iners* and decreased *L. crispatus* was seen in patients with culturable *Candida*. This suggests that even commensal carriage of *Candida* in the vagina, estimated to be >20%, could cause shifts in the microbiome predisposing patients to RVVC onset (Barousse et al., 2004). These findings are contrary to a previous clinical study showing patients who were absent for *L. crispatus* and *L. jensenii* were more often found to be culture positive for *Candida* compared with those who were *L. iners* negative. However, this could be due to geographical variations in prevalent vaginal *Lactobacillus* species as this study assessed samples from Kenyan and American women (Eastment et al., 2020). The findings in this chapter are consistent with previous studies which show the inhibitory properties of H₂O₂-producing *Lactobacillus* against *Candida* (Parolin et al., 2015, Jang et al., 2019). To investigate the significance of these findings, longitudinal clinical studies assessing the microbiome of patients before and after RVVC onset are required.

Analysis of the microbiome with respect to the form of contraceptive used by the patient revealed additional potential predisposing factors to RVVC. A notable shift in the microbiome to favour *L. iners* and lower other *Lactobacillus* species including *L. crispatus* was observed in those using both contraceptive devices and no contraception, this microbial shift was not observed in patients with RVVC using hormonal contraceptives. This proposes hormonal contraceptives may be less dysbiotic to the vaginal flora or have the potential to maintain a more health-like microbiome than contraceptive devices. Although important features were predicted at genus-level, given how slight the differences were between microbiomes it would

likely be impractical to attempt to distinguish contraceptive types based on this data. However, important features predicted at species-level found health-associated organisms like *A. vaginae* to be higher in healthy patients and those using hormonal contraceptives. Conversely, *L. iners*, associated with a dysbiotic microbiome was found to be lower in these patient groups. Although onset of VVC has been linked to increased oestrogen levels, no defined link between use of hormonal contraceptives and VVC has been identified (Geiger and Foxman, 1996). For this reason, it has been suggested that women suffering from frequent VVC episodes may wish to consider using a progestogen-only alternative (Dennerstein, 1986). However, the findings of this study suggest a more protective quality of hormonal contraceptives. Conversely, no protection was observed in women using contraceptive devices such as intrauterine devices (IUDs). Previous studies have shown IUDs provide a reservoir for *Candida* biofilm formation *in vivo* which may facilitate persistence of RVVC (Auler et al., 2010, Chassot et al., 2008). To investigate whether hormonal contraceptives have protective properties to maintain vaginal homeostasis, longitudinal studies of the vaginal microbiome are required. One study observed variations in the vaginal microbiome following introduction of hormonal contraceptives over 6 months, with respect to BV-associated microbes (Achilles et al., 2018). This study identified an increased prevalence of BV-associated bacteria in women with copper IUDs, however hormonal contraceptives did not shift the microbiome towards disease. Additionally, a robust meta-analysis identified a negative correlation between the use of hormonal contraceptives and the occurrence and recurrence of BV (Vodstrcil et al., 2013). Further studies monitoring the vaginal microbiome before and after RVVC treatment are required to determine whether hormonal contraceptives provide protection against dysbiosis.

Analysis of the microbiome with respect to how recently patients had received antifungal treatment revealed a potential risk of recurrence of disease. This analysis found women who were treated within one week of sampling to have a microbiome more similar to healthy women. However, women treated over one week before sampling were found to have a more dysbiotic microbiome associated with higher levels of *L. iners* and lower levels of *Atopobium vaginae* and *L. crispatus*. Similarly, a gradual decline towards dysbiosis was seen over time with RVVC. An intermediate profile in patients with disease for <6 months to a more severe dysbiosis in patients

with RVVC for >6 months was observed. Patients with disease for >6 months showed a higher association with *L. iners* and *Bifidobacterium* species and a lower association with *L. crispatus*, *L. jensenii* and *A. vaginae* than women with RVVC for longer. This data suggests that, although somewhat effective, the current antifungal treatments for RVVC do not alter the microbiome to a sufficient level to reintroduce a health-like state. This results in the microbiome reverting to favour a disease-like environment after one month. This is similar to results found by another author investigating the microbiome of VVC patients before and after treatment with fluconazole; The authors reported a transition to a state between disease and health following treatment as opposed to returning to a healthy microbiome (Liu et al., 2013b).

Although not associated with vaginal dysbiosis, the genus *Shuttleworthia* was positively correlated with *Candida* load and increased time between treatments and is often observed in high levels in vaginal microbiome studies (Manzoni et al., 2006). Regardless of disease status, *Lactobacillus* was negatively correlated with the presence of *Candida* as well as increased numbers of episodes and time between treatments, indicating the potential protection from *Candida* overgrowth provided by vaginal lactobacilli. It is becoming more widely recognised that current treatments for RVVC are insufficient and may even exacerbate symptoms and length of disease, however, there remain few options for women with debilitating recurrent cases. In this disease, it is important to consider both the microbiome of each individual patient as well as the *Candida* isolate before appropriate treatment should be administered. The prospect of bench-side sequencers and personalised medicine could greatly improve treatment options as well as reduce the high rates of recurrence seen in VVC episodes.

It is hypothesised that changes within *Candida* allow for it to shift from asymptomatic commensal to pathogenic yeast in the vaginal environment. It may now be more important to study RVVC with specific focus on the microbes present during RVVC, specifically *Lactobacillus*, and interkingdom interactions which may influence this pathogenic shift in *Candida*. The efficacy of *Lactobacillus* probiotics to treat RVVC have been shown in various clinical studies (Murina et al., 2014, Wang et al., 2017, Vladareanu et al., 2018). A study to investigate both the microbiome and mycobiome

of healthy women and those with RVVC before and after treatment could elucidate important interkingdom interactions in the disease. Further, it could identify candidates for probiotic therapies as well as molecular targets in *Candida* which could be targeted with future antifungals. By investigating these protective vaginal *Lactobacillus* species to gain a deeper understanding of their role in maintaining vaginal health, it may be possible to determine a suitable probiotic therapeutic to effectively treat RVVC and re-establish a healthy microbiome.

Chapter findings

- There is not an overall reduction in the abundance of *Lactobacillus* during RVVC compared with health, however, the functional capacity of key organisms is lost during disease as a result of a reduction in protective *Lactobacillus* species including *L. crispatus* and *L. jensenii*. Additionally, the presence of high quantities of *L. iners* in the vaginal environment indicates a dysbiotic microbiome.
- Although more appropriate analysis tools are required before it should be used for clinical studies, the Oxford Nanopore Technologies MinION may be more suited to the study of diseases that require accurate species-level identification than the Illumina MiSeq platform.
- Women with RVVC using hormonal contraceptives have a more similar bacterial profile to healthy women, suggesting they may provide protection from dysbiosis through maintenance of the microbiome during disease.
- The importance of the interactions of *Lactobacillus* and *Candida* in RVVC research has been underappreciated and a deeper understanding of how certain *Lactobacillus* species maintain health could greatly improve future treatment options through the use of *Lactobacillus* probiotics.

5 *Lactobacillus crispatus* exploits *Candida albicans* gene expression to facilitate its own growth

5.1 Introduction

The causative organism of VVC, *Candida spp.*, are biofilm-forming, opportunistic pathogenic yeasts with a commensal carriage rate in pre-menopausal women of up to 33% (Achkar and Fries, 2010a). Reportedly, VVC will affect 75% of women during their child-bearing years with *C. albicans* isolated in up to 90% of cases (Linhares et al., 2001, Sobel, 2007). Frontline antifungal treatments for VVC include the use of various azoles, however, resistance and subsequent recurrence of disease is well documented (Sobel, 2016, Whaley et al., 2016). Despite this, the interactions within the vaginal environment which allow for *Candida spp.* to become pathogenic, initiating VVC onset, are yet to be determined.

The predominant bacterial taxa in the vaginal environment is *Lactobacillus*, namely *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* (Petrova et al., 2015). Dysbiosis of the vaginal environment is often attributed to a loss or reduction in lactobacilli species which, coupled with an increase in vaginal pH, is thought to allow overgrowth of pathogenic microbes such as *Gardnerella vaginalis* in bacterial vaginosis (BV) and *C. albicans* in VVC (Liu et al., 2013b, Oakley et al., 2008). In healthy women, lactic acid bacteria (LAB) are thought to be responsible for maintaining a homeostatic microbiome by inhibiting growth and adhesion of other microbes via the production of secreted metabolites such as lactic acid, biosurfactants, bacteriocins and hydrogen peroxide (H₂O₂) (Figure 5.1). However, the mechanisms behind the influence these metabolites have on *Candida* remains largely unknown.

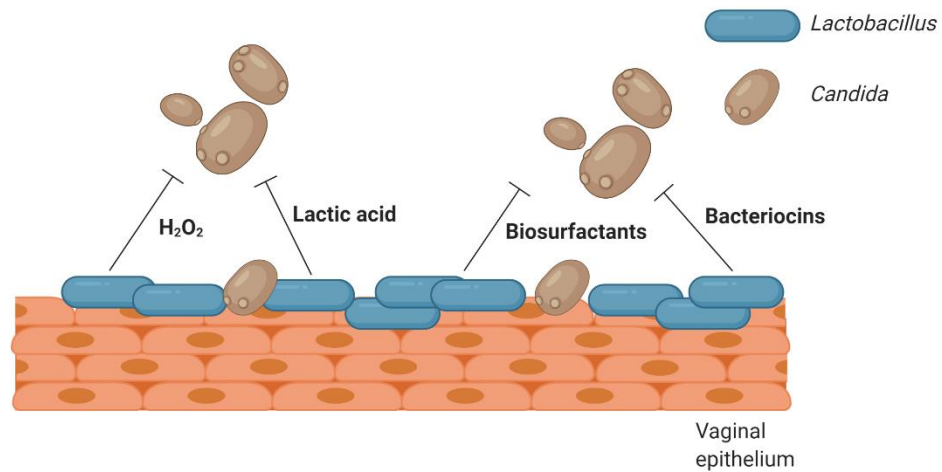


Figure 5.1: Inhibition of *Candida* adhesion to vaginal epithelium by *Lactobacillus*.
Created with BioRender.

L. crispatus is a prominent commensal of the healthy vaginal environment in various microbiome studies and produces both lactic acid and H_2O_2 (Reid, 2018, van der Veer et al., 2019). Additionally, *L. crispatus* secretes the L-lactic acid isomer which has been extensively studied for its ability to lower vaginal pH, elicit anti-inflammatory responses and inhibit microbial colonisation (Hearps et al., 2017, O'Hanlon et al., 2013). H_2O_2 is an oxidising agent which is toxic to catalase-negative bacteria and *C. albicans* (Eschenbach et al., 1989). It is thought that women with high levels of H_2O_2 -producing lactobacilli species (*L. acidophilus*, *L. crispatus* and *L. jensenii*) gain a degree of protection against herpes simplex virus type 2 and BV (Matu et al., 2010, Conti et al., 2009). However, H_2O_2 is not required by *Lactobacillus* to inhibit *C. albicans* as the non- H_2O_2 producing *L. rhamnosus* GR-1 strain has been shown to inhibit both *C. albicans* and *C. glabrata* (Kohler et al., 2012, Chew et al., 2015). Additionally, the protective role of H_2O_2 against infection is controversial in the hypoxic vaginal environment as it is produced predominantly under aerobic conditions (Tachedjian et al., 2017).

Candida and lactobacilli species are often found to co-inhabit various niches throughout the human body including the oral cavity, gut and vagina (O'Donnell et al., 2015, Mason et al., 2012, Zhou et al., 2009a). Although antagonism between *L. crispatus* and *C. albicans* has been well documented, showing a reduction in both *Candida* growth and hyphae formation, the specific genes and pathways responsible for these observations remain undefined (Jang et al., 2019, Matsuda et

Chapter 5 – *Lactobacillus crispatus* exploits *Candida albicans* gene expression to facilitate its own growth

al., 2018a, Wang et al., 2017). One study assessing the effects of cell-free supernatants (CFS) of a vaginal isolate of *L. crispatus* against *C. albicans* noted downregulation of hyphae-specific genes including *ALS3*, *HWP1* and *ECE1* (Wang et al., 2017). These findings support the probiotic potential of *L. crispatus* to inhibit *C. albicans* biofilm formation and prevent infection in the vaginal environment. Notably, these studies focus solely on the effects of *Lactobacillus* CFS on *C. albicans*, potentially ignoring crucial direct interactions between the two microbes. Furthermore, a caveat of these studies is the lack of powerful RNA sequencing technologies with the potential to expose novel mechanisms and pathways by which *C. albicans* may be inhibited by *Lactobacillus*. Advances in these technologies have led to them being more affordable to smaller labs, allowing for the production of large data sets analysing genome-wide transcripts.

In the era of antibiotic and antifungal resistance, the use of lactobacilli species as probiotics for the treatment of *Candida* infections has become increasingly attractive (Matsubara et al., 2016a). Probiotics are defined by the World Health Organisation (WHO) as “live microorganisms that are administered in adequate amounts and confer a health benefit on the host”. Studies have investigated the probiotic potential of commercially available lactobacilli species including *L. rhamnosus GR-1* and *L. reuteri RC-14* against *C. albicans* as a therapeutic for VVC (Chew et al., 2015, Jorgensen et al., 2017, Matsubara et al., 2016b, Lourenco et al., 2018). Although these studies have shown some success with impeding *C. albicans* growth and hyphal formation, these species are rarely reported as commensals of the vaginal environment and are only capable of transient colonisation during treatment (Colodner et al., 2003). Additionally, the molecular mechanisms by which these microbes are able to inhibit *C. albicans* remain largely undefined.

Hypotheses and aims

Although it is becoming more common to consider *Lactobacillus* probiotics as potential therapies for *Candida* infections such as oral and vaginal candidiasis, the mechanisms behind antagonism between *Candida* and *Lactobacillus* remain largely unknown. A better understanding of these interkingdom interactions at a molecular level may be crucial to unearthing potential targets for much-desired therapeutics. Therefore, the aims of this chapter were to:

- Investigate antagonism between *C. albicans* and commonly isolated vaginal *Lactobacillus* species.
- Elucidate the intimate interactions of the most common causative organism in VVC, *C. albicans*, and one of the most common bacterial species associated with vaginal health, *L. crispatus*, in a biofilm co-culture model.
- Observe differential expression of specific gene pathways in *C. albicans* as a result of *L. crispatus* co-culture.

It is hypothesized, based on previous studies, that antagonism between the two microbes will result in *C. albicans* expressing genes associated with stress, possibly partially due to lactic acid. Additionally, it is thought that this transcriptome analysis may provide potential mechanisms by which *L. crispatus* could contribute to resolution of VVC. To our knowledge, this is the first study to analyse *C. albicans* transcripts following biofilm co-culture with *L. crispatus* using RNA sequencing technology.

Work from this chapter has been published in mSystems:

McKloud E, Delaney C, Sherry L, Kean R, Williams S, Metcalfe R, Thomas R, Richardson R, Gerasimidis K, Nile CJ, Williams C, Ramage G. Recurrent Vulvovaginal Candidiasis: A Dynamic Interkingdom Biofilm Disease of *Candida* and *Lactobacillus*. mSystems. 2021 Aug 10:e0062221. doi: 10.1128/mSystems.00622-21.

5.2 Materials and Methods

5.2.1 Strain and culture conditions

Candida albicans SC5314, *Lactobacillus crispatus* ATCC 33820, *L. casei* ATCC 393, *L. fermentum* ATCC 14931, *L. iners* DSMZ 13335, *L. salivarius* ATCC 11741, *L. jensenii* ATCC 25258 and *L. rhamnosus* ATCC 7469 were used in this chapter. *C. albicans* was cultured on Sabouraud's Dextrose (SAB) agar for 48h at 30°C. *L. crispatus* and *L. iners* species were cultured anaerobically on De Man Rogosa and Sharpe (MRS) agar for 48h at 37°C, whilst the remaining lactobacilli species were cultured in a 5% CO₂ atmosphere. For biofilm formation, overnight cultures of *C. albicans* and *Lactobacillus* were grown in YPD and MRS media, respectively, under appropriate culture conditions. Cultures were washed twice with PBS and standardised in 1:1 medium to 1x10⁶ for *C. albicans* and 1x10⁷ for *L. crispatus*.

5.2.2 Media preparation

THB was supplemented with 10µM menadione and 10µg/mL hemin (ThermoFisher) and mixed 1:1 with RPMI (referred to as 1:1 medium).

5.2.3 Whole-genome transcriptional analysis

5.2.4 Antagonism between *C. albicans* and *Lactobacillus* in co-culture

The ability of 7 *Lactobacillus* species to inhibit *C. albicans* SC5314 biofilm formation was assessed. Overnight cultures were grown and standardised as described above. Eight biofilms of each *C. albicans*-*Lactobacillus* pair were incubated in 5% CO₂ for 24h. Alternatively, *C. albicans* biofilms were formed for 4h prior to addition of *Lactobacillus* for 20h before biomass was quantified using the crystal violet assay (Feoktistova et al., 2016). Briefly, biofilms were allowed to dry before wells were flooded with 0.05% crystal violet and incubated at room temperature for 20 min. Biofilms were then washed with water to remove excess dye before bound dye was removed with 100% ethanol and absorbance read spectrophotometrically at 570nm.

To assess *C. albicans* biofilm-related gene expression in the presence of *Lactobacillus*, RNA was extracted from 24h dual species biofilms using the PureLink RNA mini kit (ThermoScientific, Loughborough, UK), following manufacturer's

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instructions. 2µg of RNA was then converted to cDNA using the high capacity cDNA reverse transcription kit (ThermoScientific, Loughborough, UK) and 1µl used in a 20µl real-time qPCR reaction with 10µl 2X Fast SYBR® Green PCR Master Mix, 1µl forward/reverse primer and UV-treated H₂O. Primer sequences can be found in Table 1. Gene expression was analysed in duplicate on three separate occasions, no reverse transcription (NRT) and no template controls (NTC) were included throughout. Gene expression was normalised to the *ACT1* housekeeping gene and calculated using the $\Delta\Delta C_t$ method.

Table 5.1: qPCR primer sequences

Gene name	Primer sequence (5' – 3')
<i>HWP1</i>	F - GCTCAACTTATTGCTATCGCTTATTACA R - GACCGTCTACCTGTGGGACAGT
<i>ECE1</i>	F - GCTGGTATCATTGCTGATAT R - TTCGATGGATTGTTGAACAC
<i>ALS3</i>	F - CAACTTGGGTTATTGAAACAAAAACA R - AGAAACAGAAACCCAAGAACAACCT
<i>ACT1</i>	F - AAGAATTGATTTGGCTGGTAGAGA R – TGGCAGAAGATTGAGAAGAAGTTT
<i>C. albicans</i>	F – GGGTTTGCTTGAAAGACGGTA R – TTGAAGATATACGTGGTGGACGTTA

5.2.5 *In vitro* transcriptomic analysis of *C. albicans* interactions with *L. crispatus*

Overnight cultures were standardised in 1:1 broth as described above, and *Candida* biofilms grown in t75 cell culture flasks (Corning, USA) for 4 h in 5% CO₂. After incubation, media was removed and biofilms washed with PBS before *L. crispatus* was added for an additional 2, 4 or 20 h. At each time point the media was removed and biofilms washed with PBS before being imaged to ensure no contamination and scraped in to 1 mL of RNA_{later} (ThermoScientific, Loughborough, UK). Spent media from biofilms was retained and pH monitored throughout the experiment using a pH meter. A graphical illustration of the experimental methodology can be found in Figure 5.2.

5.2.6 RNA extraction from *C. albicans*/*L. crispatus* biofilms

RNA was extracted from microbial biofilms using the RiboPure™ RNA Purification Kit for yeast (ThermoScientific, Loughborough, UK), following manufacturer's instructions. Biofilms were centrifuged at >12,000g for 5 min to pellet cells and supernatants were discarded. To each sample, 480µL lysis buffer, 48µL 10% SDS and 480µL of Phenol:Chloroform:IAA were added before the pellet was resuspended by vortexing and the full mixture transferred to bead beating tubes containing 750µL of ice-cold zirconia beads. Samples were then beaten for 3x30 sec cycles using a BeadBug™ microtube homogenizer (Merck, Gillingham, UK) and centrifuged at 16,100g for 5 min to separate the RNA containing aqueous layer. The aqueous layer was then transferred to a sterile tube before 1.9mL of binding buffer and 1.25mL of 100% ethanol was added and gently mixed. The entire sample was then passed through a filter cartridge placed in a collection tube and the eluate discarded. The filter was then washed with 700µL of wash solution 1, followed by washing twice with 500µL of wash buffer 2/3. The filter cartridge was then transferred to a fresh collection tube and 25µL of pre-heated elution solution added before the sample was centrifuged for 1 min to collect RNA. Finally, a DNase treatment reaction was assembled and added to each sample for 5 min. the reaction was then pelleted and purified RNA transferred to a sterile Eppendorf tube before being stored at -80°C until analysis. Integrity of RNA was assessed using a Bioanalyser system (Aligent, USA) to ensure highly intact RNA was sent for sequencing analysis. Samples were deemed acceptable if they obtained a minimum RNA integrity number (RIN) of 7.0 and a minimum quantity of 2.5µg. Genome-wide *Candida* transcripts were sequenced using the Illumina NOVASeq6000 sequencing platform (Edinburgh Genomics) to obtain 100bp paired-end reads.

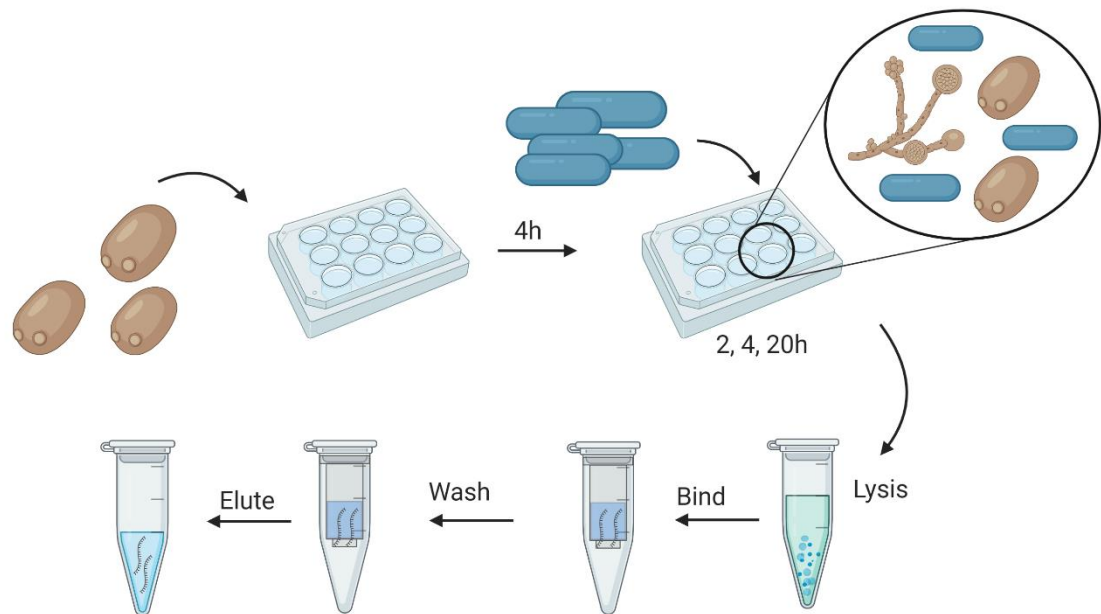


Figure 5.2: Experimental design for transcriptomic analysis. Created with Biorender.com.

5.2.7 Analysis pipeline

Genome-wide *Candida* transcripts were sequenced using the Illumina NOVASeq6000 sequencing platform (Edinburgh Genomics). FastQC and trimmomatic were implemented to score quality of raw fastq reads and remove adaptors, primers and poor-quality reads with a Phred score <30 (Bolger et al., 2014). High quality reads were aligned to the *C. albicans* SC5314 genome (Candida Genome database) using HISAT2. Aligned reads that mapped to features on the genome were enumerated using HTSeq and counts imported into R for differential expression analysis based on the DESeq2 package. Dr Christopher Delaney (University of Glasgow) performed quality control, sequence trimming and read alignment and enumeration. A summarised illustration of the bioinformatics pipelines can be found in Figure 5.3. Figures were constructed and statistical analysis performed using R, GraphPad Prism 8 and Biorender.

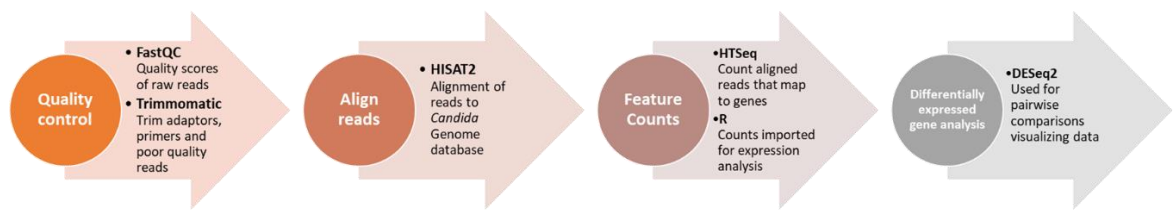


Figure 5.3: Bioinformatic pipeline for transcriptomic analysis. Created with Biorender.com.

5.2.8 Investigating the probiotic potential of *L. crispatus* in a complex biofilm model

Mature 11-species biofilms were formed as described previously by our group with slight modifications (Sherry et al., 2016). The model included the following type strains: *Streptococcus mitis* NCTC 12261, *Streptococcus intermedius* ATCC 27335, *Streptococcus oralis* ATCC 35037 and *Aggregatibacter actinomycetemcomitans* OSM 1123, *C. albicans* 3153A, *Fusobacterium nucleatum* ATCC 10596, *F. nucleatum* ssp. *vincentii* ATCC 49256, *Actinomyces naeslundii* ATCC 19039, *Veillonella dispar* ATCC 27335, *Prevotella intermedia* ATCC 25611 and *Porphyromonas gingivalis* W83. Overnight broths of each organism were standardised in 1:1 medium prior to addition to the biofilm on hydroxyapatite (HA) discs. For probiotic treatment, each biofilm was treated bi-daily (12-hour intervals) with 5×10^7 CFU/mL of *L. crispatus* for 5 mins before treatment was removed, biofilms washed 3 times in PBS and fresh media replaced. On day 3, after 4 probiotic treatments, 48h biofilms were removed for compositional analysis. On day 5, after 8 probiotic treatments, 96h biofilms were removed for compositional analysis. At each timepoint, biofilms were washed 3 times and sonicated in 1mL of PBS at 35 KHz for 10 min to remove biomass. Sonicates were split in two and to one sample 5µl of 10mM propidium monoazide (PMA) was added to quantify live *C. albicans* DNA. The other sample allowed for quantification of total *C. albicans* DNA per biofilm. All samples were incubated in the dark for 10 min then placed on ice and exposed to a 650W halogen light for 5 min. DNA was then extracted using the QIAmp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK). To a 20µl qPCR reaction, 1µl of biofilm DNA was added to: 10µl Fast SYBR™ Green Master Mix, 1µl of 10µM *C. albicans* forward/reverse primers and UV-treated nuclease-free water. Primer sequences can be found in Table 1. The following

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thermal profile was used: 95°C for 2 minutes, 40 cycles of 95°C for 3 seconds followed by 55°C for 30 seconds. Samples were assessed in duplicate from 2 separate experiments. Standard curves constructed from serially diluted DNA of *C. albicans* SC5314 were used extrapolate *Candida* colony forming equivalents (CFE/mL), respectively as described previously (O'Donnell et al., 2015).

5.2.9 Statistical analysis

Transcriptome pipeline figures were constructed using BioRender and differential gene expression plots using the DESeq2 package in RStudio. Gene ontology networks were constructed using ClueGO software, available through Cytoscape (Shannon et al., 2003). All other figures and analyses were performed in GraphPad Prism (Version 8, La Jolla, California, USA). Statistical significance was measured using t-tests to compare the means of two samples or one-way ANOVA to compare the means of multiple samples. Statistical significance was achieved if $P < 0.05$.

5.3 Results

5.3.1 Investigating antagonism between *C. albicans* and vaginal lactobacilli

To assess potential antagonism between *Lactobacillus* and *C. albicans*, 7 *Lactobacillus* species were selected and their effects on *C. albicans* biofilm-formation in co-culture observed (Figure 5.4). When grown together for 24h, a reduction in biomass was observed in all *Lactobacillus* species, particularly *L. rhamnosus* (Figure 5.4A). However, when *C. albicans* was allowed to form a biofilm before addition of *Lactobacillus*, this effect was less pronounced, and for *L. iners* and *L. fermentum*, absent (Figure 5.4B). To further analyse this antagonistic effect, *C. albicans* biofilm-related gene expression was assessed after co-culture with *L. rhamnosus* and *L. iners*. *L. rhamnosus* is often studied for its probiotic effects against *C. albicans* in mucosal environments (Mailander-Sanchez et al., 2017). Conversely, high levels of *L. iners* is hypothesised to indicate vaginal dysbiosis due to loss of protective lactobacilli. The two organisms interacted with *C. albicans* differently, i.e., *L. rhamnosus* down-regulated all biofilm-related gene expression after incubation for 20h and 24h (Figure 5.4C and 5.4D). *L. iners* down-regulated expression of *ECE1* and, to a lesser extent, *HWP1* after incubation for 24h. However, when added to a pre-existing *C. albicans* biofilm, *L. iners* resulted in up-regulation of *ece1* and *ALS3*. Although *L. rhamnosus* was able to inhibit *C. albicans* biofilm formation more than the other lactobacilli species tested, *L. crispatus* was selected for further RNA sequencing analysis. This is because *L. rhamnosus* is not a commensal of the vaginal environment and is only capable of transient colonisation. Furthermore, *L. crispatus* is one of the most prevalent *Lactobacillus* species in the vaginal microbiome and its antagonism with *C. albicans* has been displayed in previous studies.

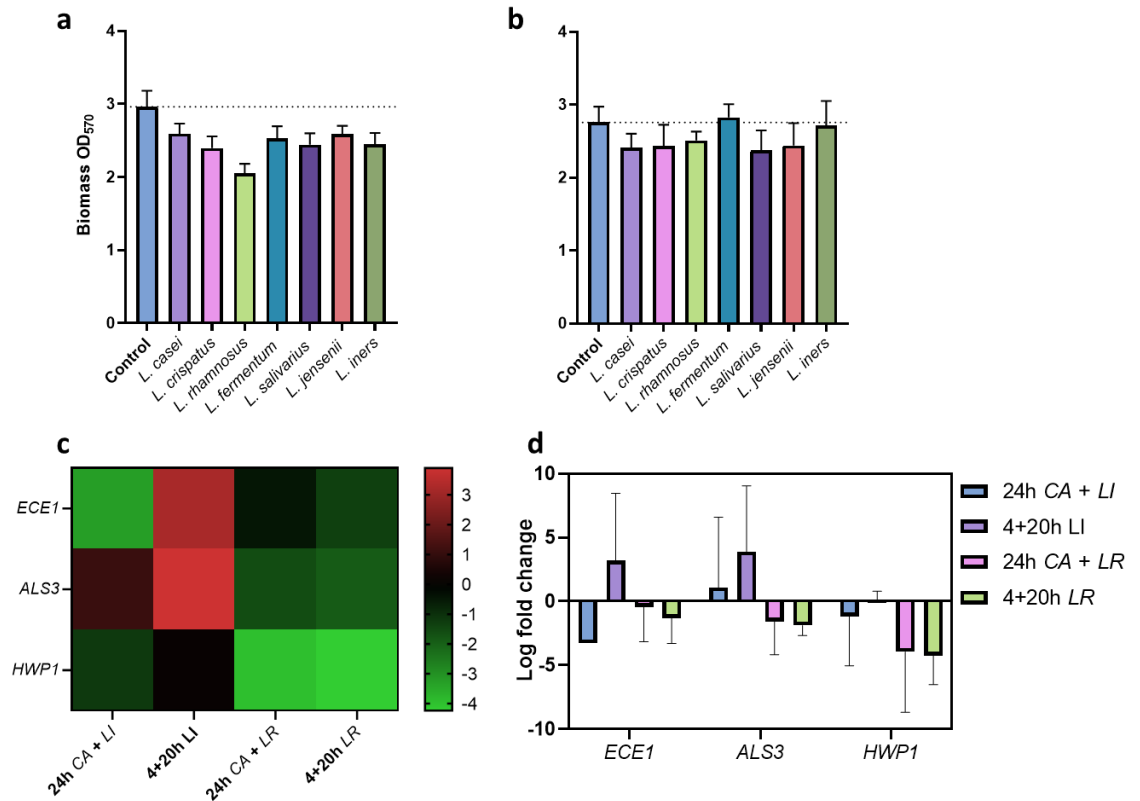


Figure 5.4: *Lactobacillus* species display antagonism with *C. albicans* in vitro. To observe inhibitory effects of *Lactobacillus* against *C. albicans* biofilm formation, *C. albicans* and a panel of *Lactobacillus* species were co-cultured together in 1:1 media in 5% CO₂ either for 24h (A) or *C. albicans* was grown for 4h prior to addition of *Lactobacillus* species for 20h (B). *C. albicans* biofilm-associated gene expression was measured in the presence *L. rhamnosus* (CA+LR), which is associated with ‘health’, and *L. iners* (CA+LI), which is hypothesised to indicate dysbiosis. The mean log fold-change relative to single species *C. albicans* biofilms is shown (C). Breakdown of expression values for gene expression analysis (D). Data represents the mean + SD.

Spent media from dual species biofilms was retained to monitor pH throughout the experiment (Figure 5.5). Both *C. albicans* single and dual species biofilms had a slightly reduced pH at 6 and 8h timepoints compared with media controls. At 24h however there was a small increase in pH in both single and dual species biofilms. Overall, the pH of the transcriptome model was relatively stable between a pH of 6.5 to 7.

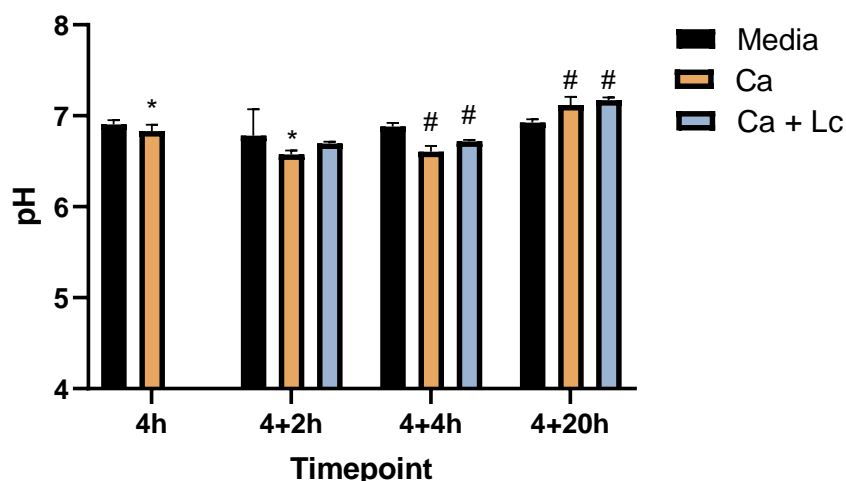


Figure 5.5: pH levels are relatively stable throughout *C. albicans* and *L. crispatus* biofilm co-culture. Overnight cultures of *C. albicans* and *L. crispatus* were standardised to 1×10^6 and 1×10^7 , respectively. *Candida* biofilms were grown for 4h before addition of *L. crispatus* for 2, 4 or 20h. After incubation, media was removed, and pH levels of media and single/dual species biofilms measured. Experiments were carried out in triplicate, on three separate occasions. Bars represent mean + SD, statistical significance compared biofilms with media controls at each time point using t-tests or one-way ANOVA (*, $P < 0.05$, #, $P < 0.0001$).

5.3.2 Differential expression analysis of co-culture model

Extracted biofilm RNA was used to assess transcriptional changes in *C. albicans* between single and dual-species biofilms with *L. crispatus*. Initially, differential expression analysis was performed in R using the DESeq2 package to observe transcriptional changes between single and dual species biofilms at each time-point. Multivariate analysis by Principal Component Analysis (PCA) showed mixed

grouping of gene expression in early single and dual species biofilms (6h and 8h) (Figure 5.6). At 24h there is separation along the y-axis of gene expression between single and dual-species biofilms, suggesting greater differences will be observed at this time point.

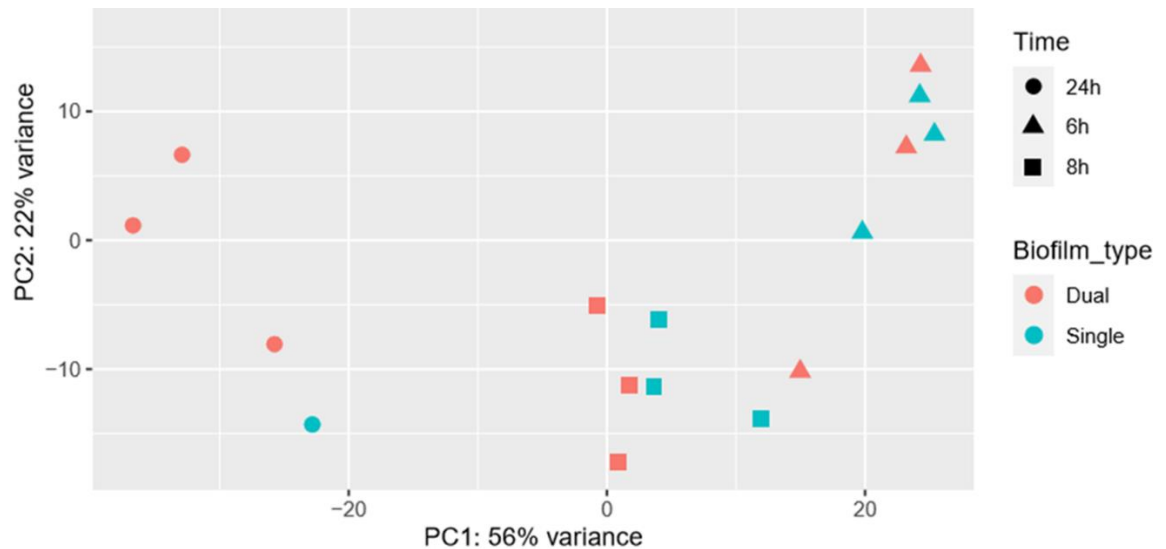
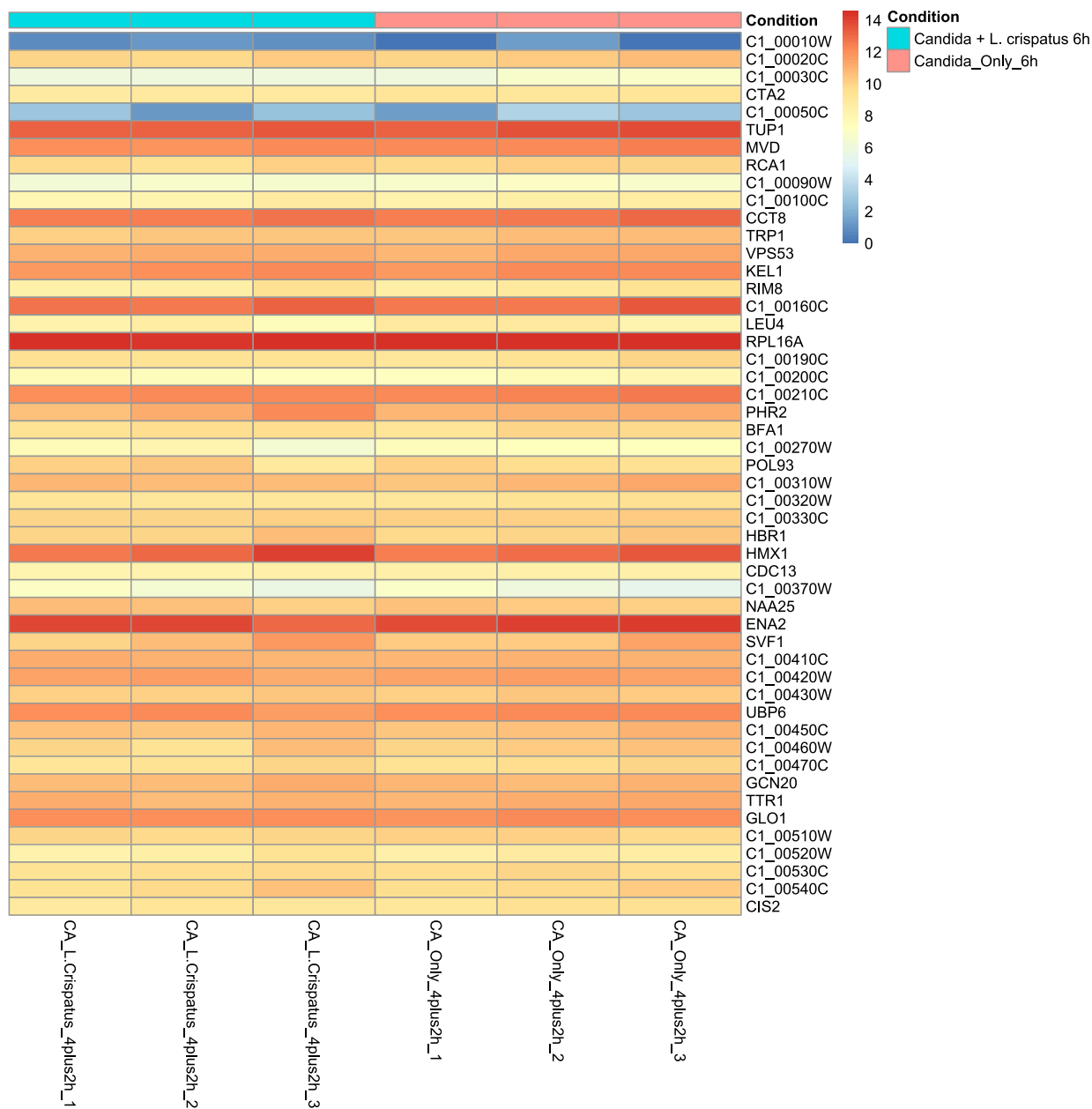


Figure 5.6: Principal Component Analysis grouping of samples based on variance in expression. PCA analysis of differential gene expression in single and dual species *C. albicans* and *L. crispatus* biofilms. PC1 shows the largest variance (56%) along the x-axis and PC2 displays the second largest variance (22%) on the y-axis

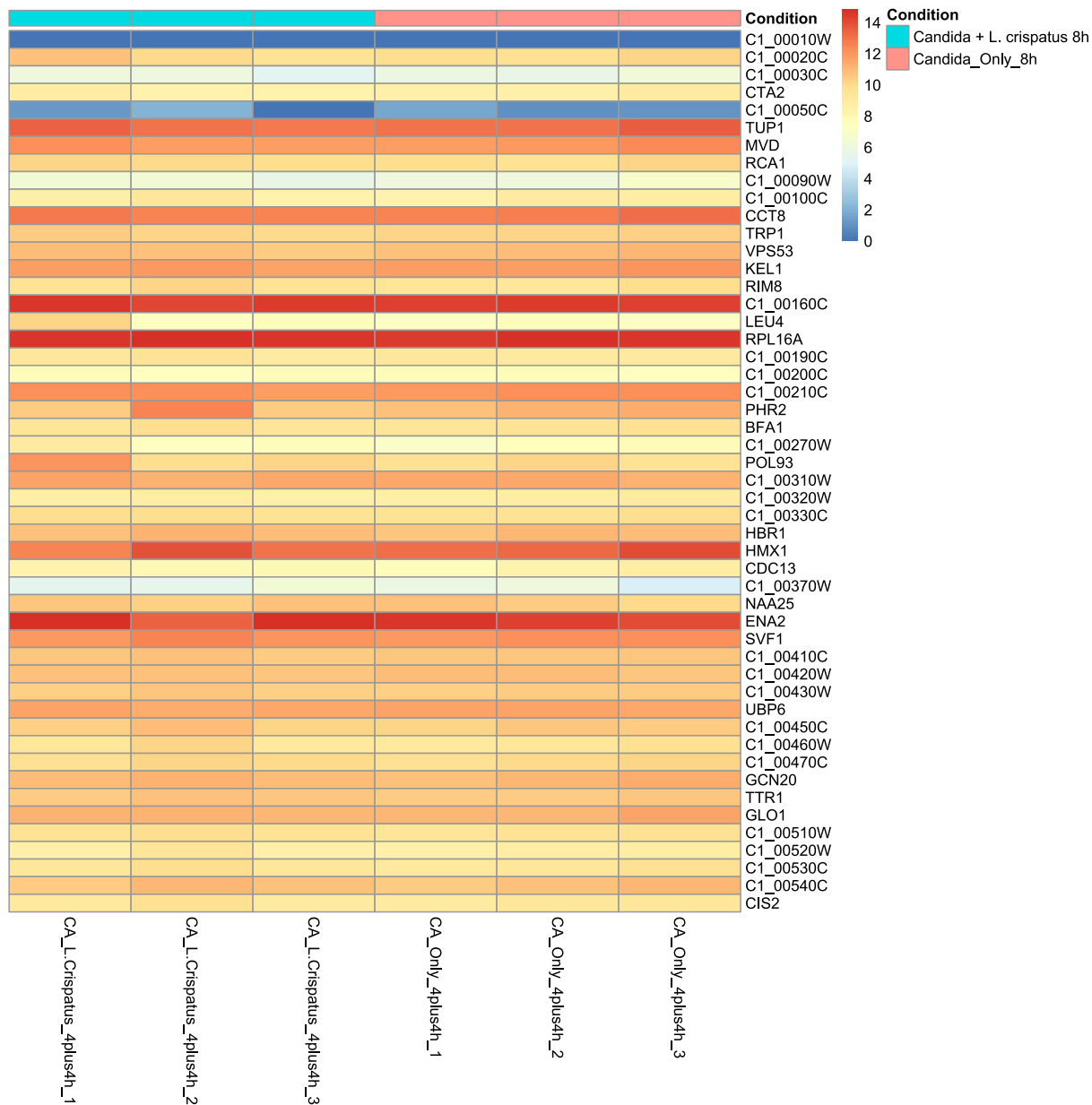
Heatmap analysis of normalised Log₂ fold change in gene expression was utilised to determine the top 50 differentially expressed genes at each timepoint (Figure 5.7). As predicted by previous analysis, expression in early dual species biofilms was similar to that observed in single species *C. albicans* biofilms (Figure 5.7A and 5.7B). However, this analysis revealed many genes involved in amino acid biosynthesis and breakdown (*ARG8*, *ILV1*, *HIS5*) upregulated in 24h dual species biofilms (Figure 5.7C). *CAR1* and *CAR2*, which are found in significantly lower levels in dual species biofilms, are involved in arginine breakdown. These data suggest it may be advantageous for *L. crispatus* to suppress expression of these genes, increasing access to arginine. Interestingly, the *PRY1* and *GTT1* genes, which are co-expressed and code for a secreted protein associated with virulence in the presence of lactate in *C. albicans* was found to be downregulated in dual species biofilms (Log₂ fold change = -5.35).

A



Chapter 5 – *Lactobacillus crispatus* exploits *Candida albicans* gene expression to facilitate its own growth

B



C

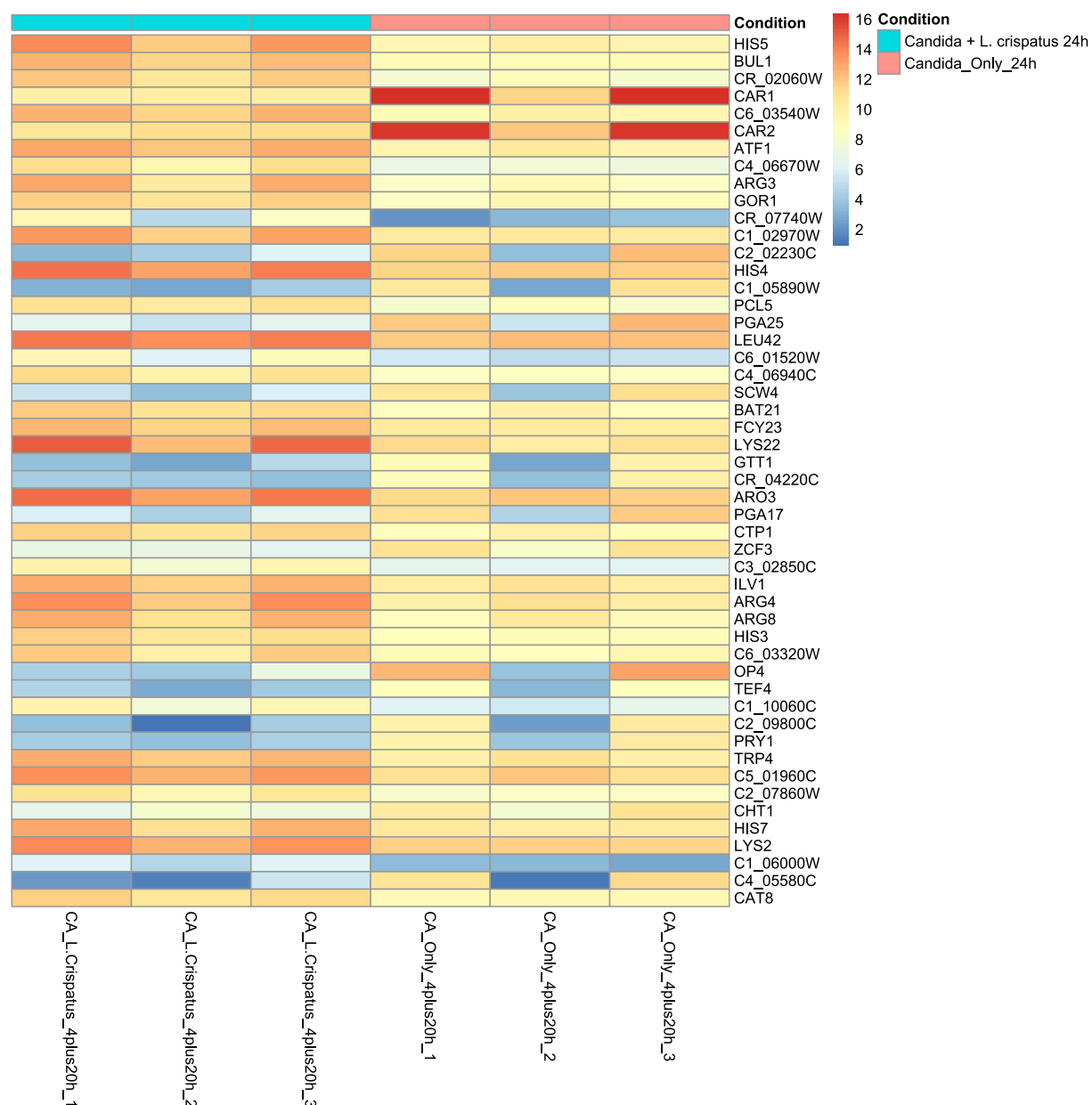
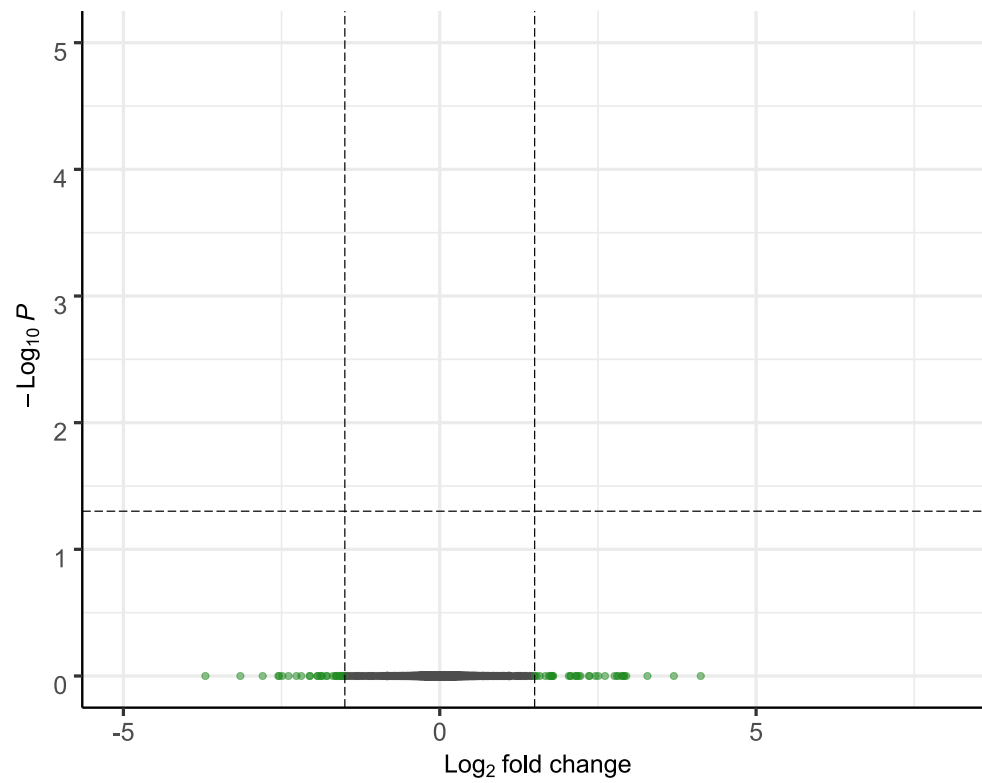


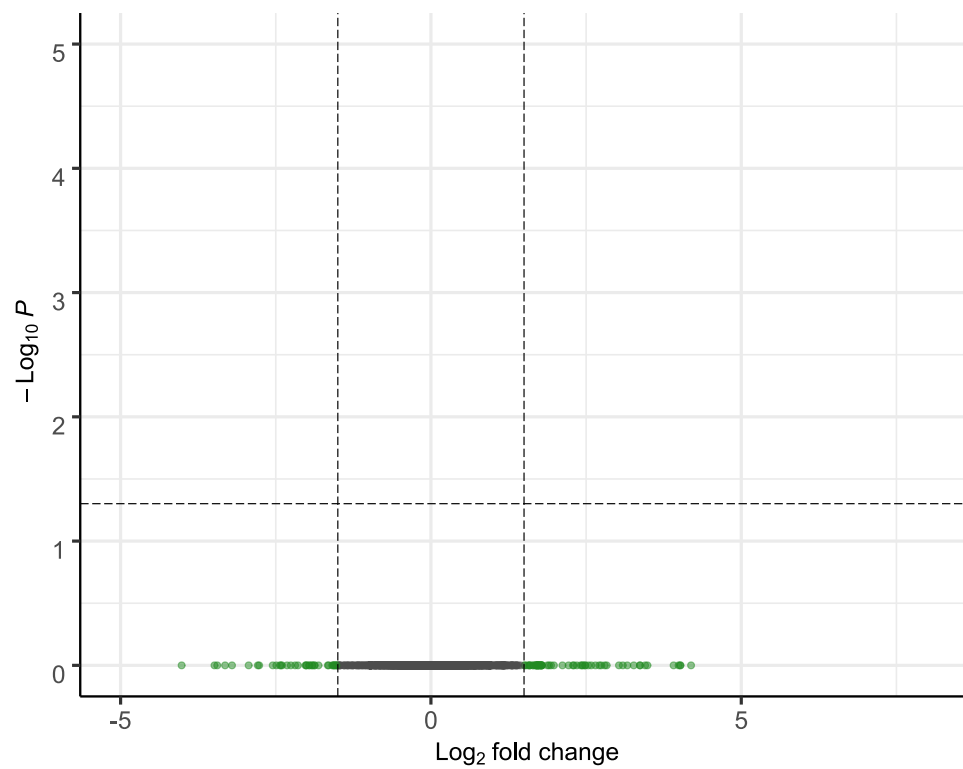
Figure 5.7: Differential expression analysis of *C. albicans* single and *C. albicans* + *L. crispatus* dual species biofilms. Heatmaps displaying the top 50 differentially expressed genes in *C. albicans* between single and dual species biofilms at 6h (A), 8h (B) and 24h (C).

To observe specific significantly differentially expressed genes between single and dual species biofilms at each timepoint volcano plots were constructed (Figure 5.8). Here it is evident that no genes in early biofilms passed the Log₂ fold change and adjusted P value cut offs for significance (Figure 5.8A and 5.8B). Subsequently, further analysis compared expression in mature 24h single and dual species biofilms only. A list of some of the key genes upregulated in 24h biofilms and their functions can be found in Table 5.2. Many of these genes are related to amino acid biosynthesis, metabolism, and breakdown as well as some involved in acetyltransferase activity and ubiquitination (*ATF1* and *BUL1*, respectively).

A



B



C

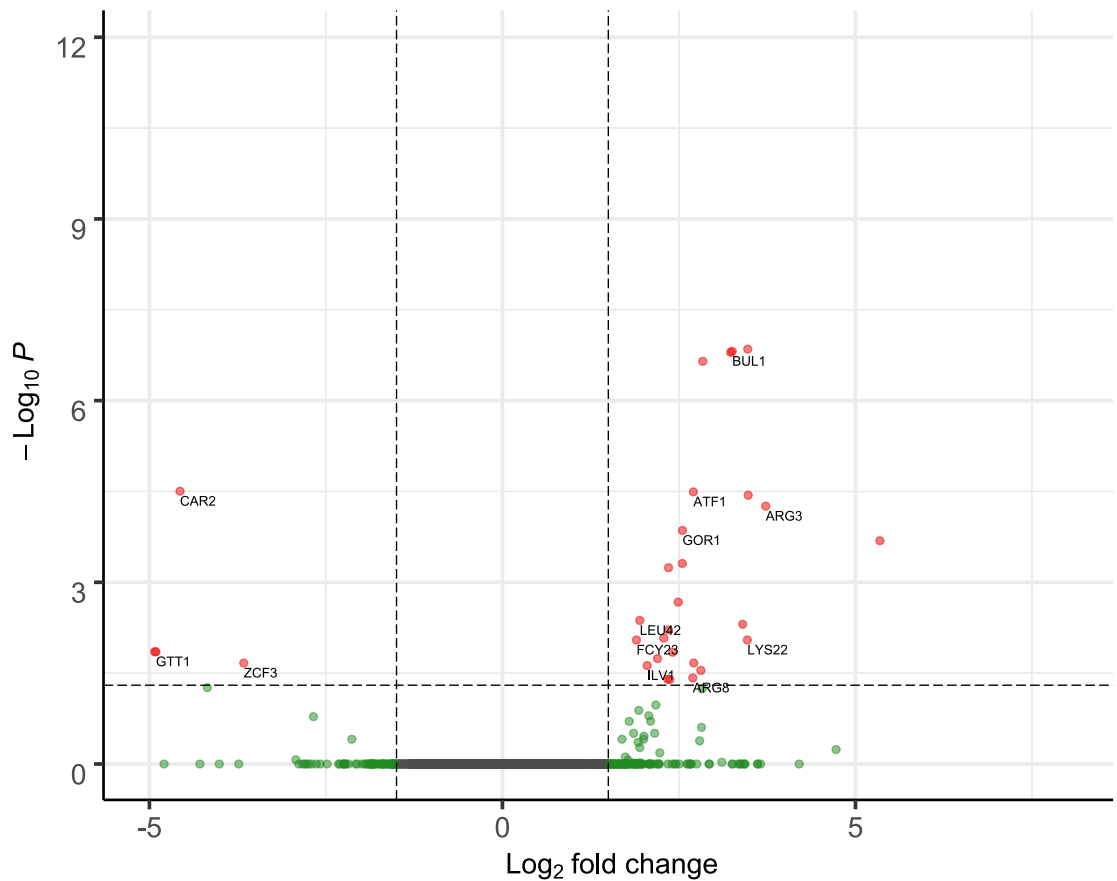


Figure 5.8: Differential gene expression occurs in mature 24h *C. albicans*/*L. crispatus* dual-species biofilms Volcano plots showing differentially expressed genes in 6h (A), 8h (B) and 24h (C) biofilms. Positive \log_2 fold change values correspond to upregulation in dual species biofilms; Negative \log_2 fold change values depict upregulation in *C. albicans* only biofilms. Significance was achieved if \log_2 fold change > 1.5 or < -1.5 (Green) and adjusted P value < 0.05 (Red).

Table 5.2: Up-regulated genes in 24h dual-species biofilms associated with amino acid biosynthesis and/or breakdown.

Gene name	Function	Log ₂ Fold change
<i>HIS5</i>	Histidine biosynthesis	3.474415
<i>BUL1</i>	Protein ubiquitination	3.254671
<i>ATF1</i>	N-acetyltransferase activity	2.704281
<i>ARG3</i>	Arginine & citrulline biosynthesis	3.728997
<i>HIS4</i>	Histidine, purine and pyrimidine biosynthesis	2.352654
<i>LEU42</i>	Leucine, valine and isoleucine biosynthesis	1.945496
<i>BAT21</i>	Leucine, valine and isoleucine biosynthesis/breakdown	2.284677
<i>LYS22</i>	Lysine biosynthesis	3.469013
<i>ARO3</i>	Phenylalanine, tyrosine and tryptophan biosynthesis	2.411573
<i>ILV1</i>	Leucine, valine and isoleucine biosynthesis, threonine breakdown	2.049845
<i>ARG4</i>	Arginine biosynthesis/metabolism	2.810158
<i>ARG8</i>	Arginine biosynthesis/metabolism	2.695885
<i>HIS3</i>	Histidine, purine and pyrimidine biosynthesis	2.340446

5.3.3 Functionality of differentially expressed genes

Gene set enrichment analysis (GSEA) was used to perform Gene ontology (GO) term analysis to determine the functionality of differentially expressed genes in single and dual species 24h biofilms. Differentially expressed genes were assigned to GO terms based on function. The 36 identified differentially expressed genes were assigned to 25 GO terms. These functions are characterised by a larger network of amino acid biosynthesis, metabolism and breakdown processes and a smaller network of transaminase processes (Figure 5.9A). Analysis of overrepresented GO terms showed an increase particularly in arginine and histidine biosynthetic and metabolic processes with >35% of associated genes upregulated (Figure 5.9B).

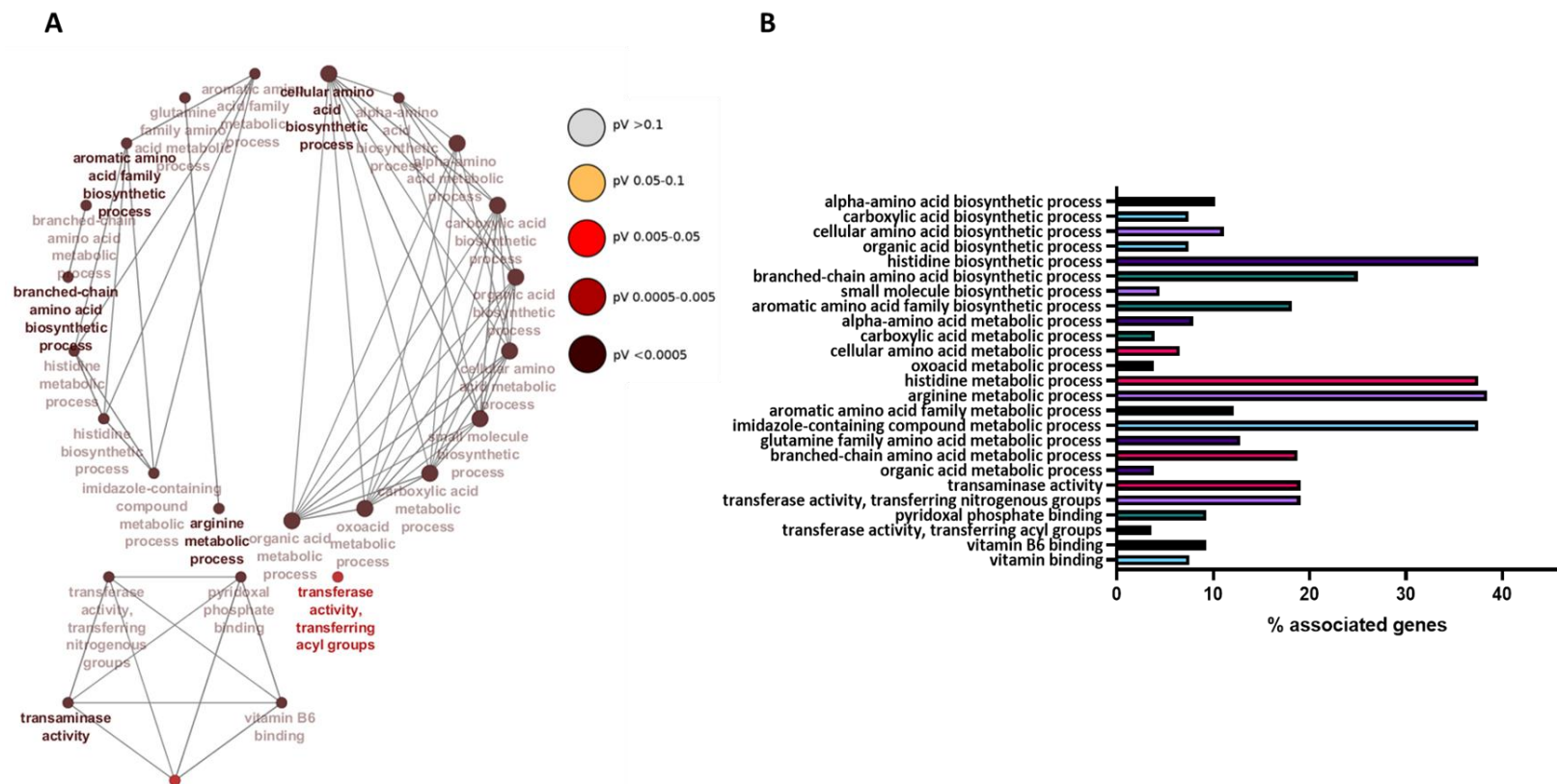


Figure 5.9: Gene networks of Gene Ontology (GO) terms and over-represented GO terms.

Constructed gene networks of GO terms in 24h biofilms (A) and significantly overrepresented GO terms in 24h dual species biofilms compared with single species (B). All GO terms have an adjusted P value < 0.05. Nodes are coloured by significance. Networks were created using ClueGO software available through Cytoscape.

Some of the key overrepresented pathways upregulated in 24h biofilms are highlighted in Figure 5.10. Despite upregulation of α -amino acid biosynthesis and metabolism pathways in *C. albicans*, expression of *BAT21* and *ILV1* in dual-species biofilms suggests *C. albicans* is in a state of amino acid starvation during co-culture with *L. crispatus* (Figure 5.10B). Additionally, genes involved in production of arginine and histidine were highly upregulated in dual species biofilms (Log_2 fold change >3).

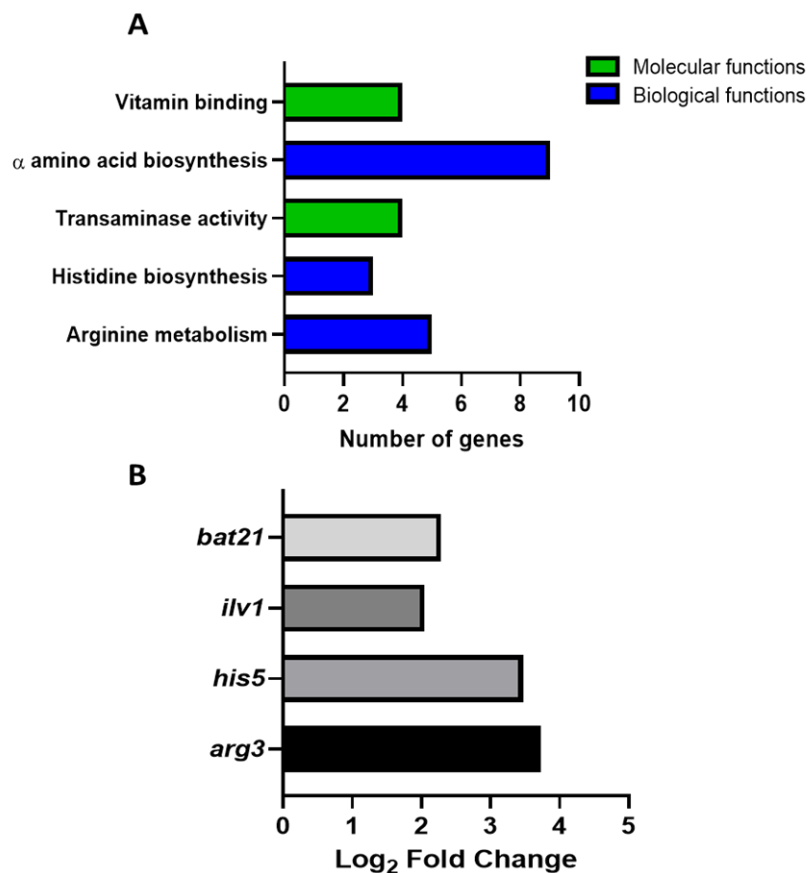


Figure 5.10: Genes involved in α -amino acid biosynthesis and breakdown are upregulated in *C. albicans*/*L. crispatus* biofilms. Important biological and molecular functions in dual species biofilms (A). Log_2 fold change of key gene expression in *C. albicans* from single to dual species biofilms (B). All GO terms have an adjusted P value < 0.05 .

5.3.4 Probiotic effect of *L. crispatus* against *C. albicans*

Given the antagonism observed between *C. albicans* and *L. crispatus*, the next aim was to investigate the *in vitro* probiotic potential of *L. crispatus* against *C. albicans* infection in a complex biofilm model (Figure 5.11). After 2 consecutive days of probiotic treatment, a slight reduction in total and live *C. albicans* composition within the biofilm is seen, however this was not significant ($P = 0.55$, $P = 0.16$, respectively) (Figure 5.11A and 5.11B). Following a 4-day treatment regimen with *L. crispatus*, total *C. albicans* composition significantly decreased ($P < 0.05$) with reduced levels of live fungal DNA. When assessing the total fold reduction in *C. albicans* from untreated biofilms, the greatest probiotic effect is observed at 48h post-treatment (Figure 5.11C).

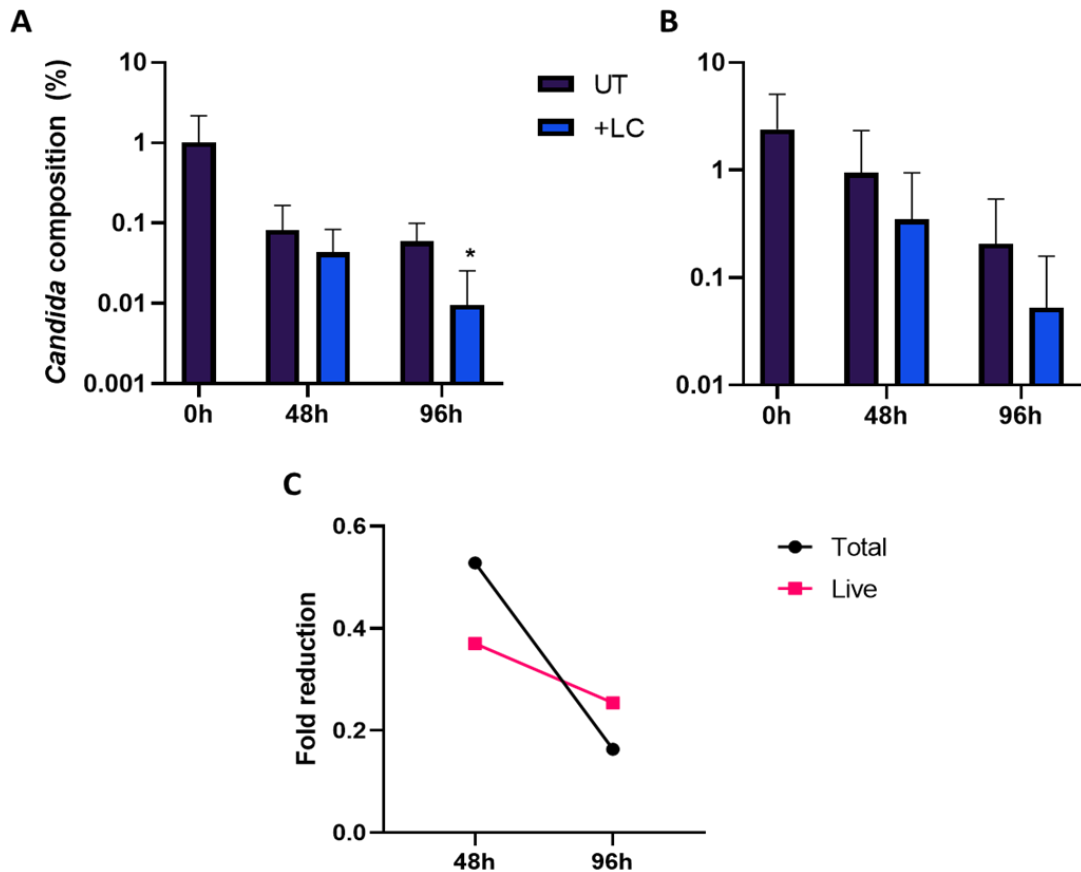


Figure 5.11: Bi-daily addition of *L. crispatus* reduces *C. albicans* load within a complex biofilm model *in vitro*. The potential probiotic properties of *L. crispatus* against *C. albicans* were assessed in a 11-species biofilm model treated twice daily with *L. crispatus*. Live/Dead qPCR allowed for quantification of percentage composition of total (A) and live (B) *C. albicans* DNA within the biofilm. Average fold change in the *C. albicans* percentage composition from the untreated 11-species biofilm is also shown (C). Data represents mean + SD. Statistical analysis compared treated and untreated biofilms at each time point using paired t-tests comparing raw CFE values (*, $P < 0.05$).

5.4 Discussion

Initially, the ability of 7 *Lactobacillus* species to inhibit *C. albicans* biofilm formation in co-culture was assessed. *L. rhamnosus* has been studied extensively for its potential as a probiotic and has been shown to prevent adhesion of *C. albicans* to mucosal surfaces (Manzoni et al., 2006). This data confirms these findings with *L. rhamnosus* displaying the greatest ability to inhibit biofilm formation. Interestingly, when added to a pre-formed *C. albicans* biofilm, the ability of all species assessed to inhibit biofilm formation is suppressed. This is likely due to the thick nature of *C. albicans* biofilm formation with secreted metabolites from *Lactobacillus* unable to penetrate the ECM and disrupt the biofilm. Although this suggests *Lactobacillus* probiotics may be ineffective against *C. albicans* biofilms, this phenomenon may not be observed in a polymicrobial *in vivo* setting where *C. albicans* biofilms are less confluent. Additionally, the ability of *L. rhamnosus* to down-regulate *C. albicans* biofilm-related gene expression was also shown. *L. iners* is isolated from the vagina of 50% of both healthy and diseased woman but, unlike other vaginal lactobacilli, is not thought to contribute to maintaining health (Petrova et al., 2017). This is due to its notably small genome compared to other species which brings researchers to assume it may be parasitic or symbiotic in nature. For this reason, high levels of this microbe are thought to increase risk of vaginal disease and potentially indicate dysbiosis. Additionally, *L. iners* results in up-regulation of both *ALS3* and *ECE1* and as such, should not be considered as a probiotic treatment for *C. albicans* infections.

L. crispatus is increasingly becoming recognised as a health-associated *Lactobacillus* species in the vaginal microbiome and may be important in preventing RVVC. As well as being associated with health, *L. crispatus* has been shown to promote stability of the vaginal microbiome in longitudinal studies (Antonio et al., 1999, Verstraelen et al., 2009). The aim of this transcriptomic experiment was to elucidate possible antagonism between *C. albicans* and *L. crispatus*. The *in vivo* vaginal pH is thought to be <4.5, however, in this dual species model the pH was sustained between 6-7. Previously, vaginal *L. crispatus* isolates have been shown to acidify growth media to levels comparable to that of the *in vivo* vaginal environment (Boskey et al., 1999). Although using different growth media, it is likely that the presence of *C. albicans* in this model is responsible for the increased pH.

Vaginal disease is often coupled with an increase in vaginal pH, due to reduction in lactobacilli, allowing for pathogens such as *C. albicans* to gain a niche. Further, this is evidence of antagonism between *C. albicans* and *L. crispatus* as *C. albicans* has been shown to neutralise the pH of growth media in order to facilitate hyphal morphogenesis (Vylkova et al., 2011). It is possible that, in the presence of other lactic acid bacteria, the low environmental pH could play a role in *Candida* pathogenesis. Nonetheless, the stability of the neutral pH throughout this experiment dictates that any transcriptomic changes observed are not due to acidification of the media and are a result of interactions between *C. albicans* and *L. crispatus*.

This study found that in a co-culture model with *L. crispatus*, *C. albicans* does not begin to display any alterations in gene expression until 24h of incubation. It has previously been shown in *Lactobacillus* clinical isolates that secreted metabolites can remain at low levels until up to 72 or 120h incubation, which may account for the delay in interaction seen here (Ogunshe et al., 2011). Out of 6,221 genes in the *Candida* genome, 26 were found to be upregulated specifically in dual species 24h biofilms. Similarly, 10 genes were found to be upregulated in single species 24h biofilms compared to dual species (Adjusted $P < 0.05$). Gene transcripts associated with amino acid biosynthesis and transaminase activity were prominent in *L. crispatus* dual-species biofilms. Transaminase activity is primarily associated with α -amino acid breakdown and biosynthesis. It has been shown in a model of *Saccharomyces cerevisiae*, *L. lactis* and *L. plantarum*, that in nitrogen rich environments yeasts secrete an array of metabolites, primarily amino acids, thereby facilitating the growth of lactobacilli (Ponomarova et al., 2017). The upregulation of various amino acid biosynthesis processes in our study may be attributed to this nutrient cross-feeding. However, this may also be a deliberate process by which *L. crispatus* is able to drive synthesis of amino acids in *C. albicans*, as well as suppressing *CAR1* and *CAR2*, associated with arginine breakdown, in order to sequester them for metabolism and facilitate its own growth. The upregulation of *BAT21* and *ILV1* in dual species biofilms is associated with amino acid starvation in *C. albicans*, suggesting that despite the upregulation of various amino acid biosynthesis processes, *C. albicans* is unable to utilise them. This may be a contributing factor in the process by which *L. crispatus* is able to out-compete

Candida during VVC and potentially re-establish a healthy microbiome. Additionally, amino acids are a key source of nitrogen for *C. albicans* and starvation of these nutrients has been shown to induce hyphal morphogenesis (Tripathi et al., 2002). It is possible that this starvation caused by *L. crispatus* contributes to the pathogenic shift in *C. albicans* and onset of VVC. Furthermore, the *PRY1* gene, associated with virulence in the presence of lactate, was found to be downregulated in dual species biofilms. Thus, *L. crispatus* may possess a mechanism by which it can suppress lactate-associated virulence in *C. albicans*. Interestingly, genes associated with resistance to lactic acid such as *mig1* and *cyb2* were not found to be differentially expressed in our co-culture study (Cottier et al., 2017, Ueno et al., 2011). This suggests a classical weak organic acid stress response in *C. albicans* is not being triggered by the presence of *L. crispatus* alone. Given that the media in this experiment was not acidified, this concludes that antagonism between *C. albicans* and *L. crispatus* is not purely due to a reduction in environmental pH. The influence of other secreted metabolites of *L. crispatus* such as H₂O₂ and bacteriocin-like substances on *C. albicans* requires further research to fully illustrate antagonism between the two organisms. These data show a potentially probiotic effect of *L. crispatus* against *C. albicans* in non-acidic environments. This antagonism is governed by the over-production of amino acids from *C. albicans* which may facilitate restoration of a healthy microbiome through lactobacilli proliferation.

This study focusses on the interactions of *C. albicans* with one *Lactobacillus* species. It may however, be beneficial for future studies to assess *Candida* gene expression in the presence of a relevant model of the vaginal microbiome *in vitro*. Additionally, this study, and many others, focuses on the interactions between *Lactobacillus* and *C. albicans*. However, in recent decades there has been a global increase in the prevalence of *non-albicans Candida* (NAC) species in VVC, up to 45%, commonly associated with antifungal resistance and recurrence (Narayankhedkar et al., 2015). To understand these more complicated cases of VVC, further studies are required to assess interactions between lactobacilli and NAC species in the vaginal environment.

Finally, the potential probiotic effect of *L. crispatus* against *C. albicans* within a complex biofilm model was investigated. Although studies have assessed the

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inhibitory effect of *L. crispatus* against *C. albicans* mono-species biofilms (Rizzo et al., 2013, Li et al., 2019, Wang et al., 2017), to our knowledge, this is the first report of *L. crispatus* reducing *C. albicans* composition within a polymicrobial biofilm model *in vitro*. This data supports other studies confirming the probiotic potential of *L. crispatus* against *C. albicans* biofilm infections. More studies are required to assess the specific mechanisms of secreted metabolites from *L. crispatus* responsible for the observed inhibition of *C. albicans* growth and biofilm formation.

Collectively, results from this chapter show a potentially probiotic effect of *L. crispatus* against *C. albicans* in non-acidic environments and within a complex polymicrobial biofilm model. This antagonism is governed by the over-production of amino acids from *C. albicans* which may facilitate restoration of a healthy microbiome through lactobacilli proliferation. Elucidating the interactions of health-associated lactobacilli with NAC species will lead to a better understanding of the interactions between *Candida* and *Lactobacillus* which allow for VVC onset and recurrence.

Chapter findings

- When added to pre-formed *C. albicans* biofilms, the probiotic effect of *Lactobacillus* species is suppressed or absent *in vitro*. *L. iners* induces biofilm-related gene expression and is not capable of *C. albicans* biofilm inhibition and as such, should not be considered as a candidate for probiotic therapies. Although *L. rhamnosus* is a viable option for probiotic treatment, its transient colonisation means other commensals of the vaginal environment should be prioritised.
- Antagonism between *C. albicans* and *L. crispatus* is present at a neutral pH. *L. crispatus* is able to drive α -amino acid biosynthesis and amino acid starvation in *C. albicans*. This may be a crucial mechanism by which *L. crispatus* is able to out-compete *C. albicans* during VVC and re-establish vaginal health.
- Bi-daily addition of *L. crispatus* to a complex polymicrobial biofilm model reduces *C. albicans* composition within the biofilm. This strengthens the viability of *L. crispatus* for further probiotic therapeutic trials in vaginally relevant biofilm models.

6 General discussion

6.1 Introduction

The work outlined in this thesis aimed to gain a greater depth of understanding of the vaginal environment and the influence of microbial interactions during health and RVVC through microbiological and metagenomic assessment of a panel of clinical samples. The literature review and bibliometric analysis outlined an increased interest in the field of *Candida* and *Lactobacillus* research in the past two decades. It also identified caveats in our knowledge and appreciation of interactions between these microbes and the potential impact of biofilm formation, particularly pertaining to vaginal research. Despite claims within the field that *Candida* does not form biofilm on vaginal mucosa, microbiological analysis performed on clinical samples revealed various indicators of *Candida* biofilm formation during RVVC. This included an increased *Candida* load following treatment as a result of resistant communities, increased tolerance to antifungals, capability of *Candida* isolates to form biofilms, and for the first time, *C. albicans* aggregate formation in lavage fluid. Analysis of the microbiome of these women revealed subtle but important differences between *Lactobacillus* species present during health and RVVC. Additionally, the hypothesis of resistant communities due to biofilm formation was strengthened where a more dysbiotic microbiome was observed in women treated over one week before sampling. This is the first study to compare the microbiome of women with RVVC with respect to length of disease, treatment received, and contraception used. Transcriptomic analysis revealed potential probiotic efficacy of *L. crispatus* against *C. albicans* biofilms in vitro in co-culture and in a complex polymicrobial setting. This is the first study to identify a potential α -amino acid biosynthesis/starvation mechanism by which *L. crispatus* may out-compete *C. albicans* during VVC/RVVC to re-establish health.

6.2 *Candida* biofilm formation in RVVC – a reservoir for failed clinical treatment?

The analysis in **Chapter 3** of this thesis utilised microbiological techniques to assess a panel of RVVC clinical samples in order to observe potential sources of failed clinical treatment reported in practice. An important outcome from this analysis was the identification of an increased fungal load in RVVC which is exacerbated in women with disease for longer. It has been postulated previously that an increased *Candida* load is indicative of symptomatic VVC/RVVC (Hopwood et al., 1988).

However, since this original study, no authors have investigated the influence of *Candida* load in clinical samples of health and VVC/RVVC on disease pathology. Although some authors have compared numbers of *Candida*-positive cultures in healthy women and those with symptomatic disease (Bauters et al., 2002), there remains very little evidence of quantitative analysis. This is likely a reflection of the current FDA-approved “gold standard” for VVC diagnosis being positive *Candida* culture, which is only 65% sensitive for accurate diagnosis (Chatzivasileiou and Vyzantiadis, 2019, Sonnex and Lefort, 1999). With the development of real-time qPCR over the last two decades as well as the increasing accessibility to species identification by 16S/18S sequencing provided by benchtop sequencers, these guidelines require revision. With improved diagnostics using qPCR or benchtop sequencers it may be possible to develop more appropriate treatments for VVC, greatly decreasing recurrence rates and the psychological and economic burdens of RVVC.

The analysis in **Chapter 3** also identified evidence of inefficient clinical treatment where women who received treatment for RVVC more than one week before sampling had a higher fungal burden than those treated within one week of sampling. Although in most cases one oral dose of 150mg of fluconazole is sufficient to treat VVC, failure to eradicate *Candida* due to insufficient treatment during VVC contributes to the 8% of women who subsequently develop RVVC (Sobel et al., 1998, Dovník et al., 2015a). Despite the debilitating consequences for women’s mental health and the economic burden of RVVC worldwide, treatment options remain limited for cases that do not respond to azole therapies. Within the last year, a novel drug for the treatment of VVC, ibrexafungerp, has been developed (Larkin et al., 2019). This drug may soon become the new frontline treatment for VVC as it has shown efficacy against various *Candida* isolates, including those resistant to fluconazole and echinocandins (Azie et al., 2020, Ghannoum et al., 2019). Additionally, it has been shown to successfully inhibit both *C. albicans* and *C. glabrata* biofilms (Marcos-Zambrano et al., 2017). Although they may not be fully accepted or even diagnosed during RVVC, this drug may allow for treatment of *Candida* biofilms in the vagina, significantly reducing rates of failed treatment and subsequent recurrence.

Finally, another key finding from this study was evidence of the presence of *Candida* biofilms in the vagina during RVVC. Although the study design did not include collection of vaginal biopsies to directly image *Candida* biofilms on vaginal mucosa, we have shown various indicators of biofilm formation. Data in **Chapter 3** shows microscopic images of *C. albicans* free-floating aggregates and hyphal formation in patient lavage fluid. Dissemination of biofilm to free-floating aggregates has been shown previously and these encapsulated communities of cells have the propensity to display increased virulence and resistance to antifungals (Uppuluri et al., 2010, Wall et al., 2019). Additionally, *Candida* clinical isolates in this study were capable of heterogeneous biofilm formation, displaying increased tolerance to azole treatments as sessile cells. This has been shown previously in clinical isolates of VVC, further strengthening the potential for these isolates to form biofilms in vivo (Sherry et al., 2017). Unlike in VVC, *Gardnerella vaginalis* biofilm formation during BV is well-defined and accepted to cause increased antimicrobial tolerance requiring more aggressive treatment (Patterson et al., 2007a, McMillan et al., 2011). Despite visualisation of *C. albicans* biofilms both in vivo and ex vivo in murine models of VVC (Harriott et al., 2010b), the presence of these biofilms is largely disputed (Swidsinski et al., 2019). Although formation of these biofilms could be a source of the failed treatment reported in practice, there are no FDA-approved treatments appropriate to diagnose or treat them. Additionally, the dismissal of their presence by authors in the field has resulted in a lack of clinical studies assessing biofilm formation in RVVC of a similar scale to those carried out for BV (Swidsinski et al., 2019). Acceptance of the presence of *Candida* biofilms on vaginal mucosa during VVC/RVVC would allow for more appropriate considerations for antifungal treatment, potentially reducing both recurrence rates and rates of development of azole-resistance in the community.

6.3 The functional capacity of *Lactobacillus* species is lost during RVVC

Analysis of the microbiome through 16S rRNA sequencing of all clinical samples in **Chapter 4** allowed for identification of subtle, key differences in bacterial species present during health and RVVC. Data comparing the two cohorts found similar *Lactobacillus*-dominated bacterial profiles at family and genus-level, with notably lower levels of H₂O₂-producing lactobacilli including *L. crispatus* and *L. jensenii* in

RVVC samples. It has been hypothesised, based on the microbiome observed during BV, that levels of *Lactobacillus* are reduced or absent during VVC (Fredricks et al., 2005). Although few studies of the vaginal microbiome during RVVC exist, they have failed to observe evidence of an altered microbiome during VVC/RVVC, disproving this theory (Zhou et al., 2009a, McClelland et al., 2009). These studies resulted in the conclusion that lactobacilli species do not contribute to protection from VVC/RVVC. However, the findings in this thesis show that although overall levels of *Lactobacillus* are not altered, the functional capacity of key species is lost during RVVC. The presence of high levels of *L. iners* seen in patients with RVVC is thought to be indicative of dysbiosis due to its lack of protective capabilities. Although very few studies have investigated bacterial species present during health and VVC, similar findings have been shown (Ceccarani et al., 2019). Protection by *L. crispatus* is owed to its high metabolic consumption of glycogen to lactic acid reducing vaginal pH, as well as its ability to produce H₂O₂, and both isomers of lactic acid (Amabebe and Anumba, 2018, Foschi et al., 2017, Witkin et al., 2013). The efficacy of *L. crispatus* to inhibit *C. albicans* growth and biofilm formation has been demonstrated (Wang et al., 2017, Jang et al., 2019). Further investigation of this interkingdom interaction using large-scale clinical studies of VVC could lead to the use of *L. crispatus* as a probiotic intervention for VVC treatment. Currently, few clinical studies assessing the microbiome of women with VVC/RVVC using 16S rRNA sequencing exist. As sequencing technologies develop and bacterial identification to species-level becomes more accurate, it may be possible to fully elucidate key *Lactobacillus* species which protect against VVC/RVVC. A greater understanding of the importance of these species would lead to a deeper knowledge of RVVC pathogenesis and potentially allow for the development of targeted treatments and sophisticated probiotic therapies. Additionally, identification of important lactobacilli would allow for investigation of interactions of these species with *Candida* to exploit mechanisms of the pathogenic switch resulting in VVC onset.

Another key finding from data in **Chapter 4** was that those suffering from RVVC using hormonal contraceptives had a bacterial profile similar to that of healthy women. The shift towards dysbiosis to favour *L. iners* and lower *L. crispatus* levels seen in women using contraceptive devices or no contraceptives was not observed in patients using hormonal preventatives. Onset of VVC is associated with increased

levels of oestrogen, suggesting a potential predisposition to disease due to hormonal contraceptives, however no clear link has been identified (Geiger and Foxman, 1996). Although it has been suggested that women suffering from frequent episodes of VVC should switch to a progestogen-only (synthetic form of progesterone) contraceptive to prevent VVC onset due to high oestrogen levels (Dennerstein, 1986), data in this study suggests potential protection from disease by hormonal contraceptives. Although literature surrounding association between contraceptive use and VVC is limited, two studies investigating the impact of contraceptives on BV onset found either no change or a negative correlation associated with use of hormonal contraceptives and disease (Achilles et al., 2018, Vodstrcil et al., 2013). Additionally, no protection from dysbiosis was observed in women using contraceptive devices. Previous studies have shown IUDs to facilitate *Candida* biofilm formation in the vagina *in vivo*, potentially predisposing these women to persistent RVVC (Chassot et al., 2008, Auler et al., 2010). The potential regulatory role of oestrogen provided by hormonal contraceptives requires further investigation to determine whether prescription of hormonal contraceptives may prevent future recurrence of vaginal diseases. Similar to the increased fungal load in patients who were treated over one week before sampling observed in **Chapter 3**, the microbiome of these women was found to be more dysbiotic than those treated more recently. Very few studies compare the vaginal microbiome before and after VVC treatment. One study observed an atypical bacterial profile somewhat similar to health following antifungal treatment, suggesting this may be an intermediate state between health and VVC (Liu et al., 2013b). Additionally, this study observed women with RVVC for longer to have more dysbiotic microbiomes. Collectively, these data suggest current antifungal therapies for VVC are insufficient to re-establish a healthy microbiome, resulting in a risk of reverting to a pathogenic profile. More studies investigating the longitudinal bacterial profiles throughout different treatment regimens are required to fully elucidate the role of the microbiome during VVC.

6.4 The importance of vaginal probiotic *Lactobacillus*

A key finding of data in **Chapter 5** was the probiotic ability of *Lactobacillus* species, particularly *L. rhamnosus*, to inhibit *C. albicans* biofilm formation. Notably, this effect was diminished or absent when added to pre-formed *C. albicans* biofilms. This may mean that *Lactobacillus* probiotics would be ineffective for VVC treatment if *C. albicans* biofilms have formed on vaginal mucosa. This data supports the use of *Lactobacillus* probiotics as an adjuvant therapy used in combination with antifungal therapies (Martinez et al., 2009, Strus et al., 2005). However, in an *in vivo* setting, *C. albicans* may not form as a confluent biofilm as seen *in vitro*, therefore making it more susceptible to probiotic interventions. Although it is not commensal to the vaginal microbiome, *L. rhamnosus* is capable of transient colonisation which may allow it to modify the microbial community to favour health (Colodner et al., 2003). Additionally, data from this chapter observed a lack of *C. albicans* biofilm inhibition by the non-protective vaginal commensal, *L. iners* (Petrova et al., 2017). Collectively, these data support the use of *Lactobacillus* probiotics, using species such as *L. rhamnosus* and *L. crispatus*, for the treatment of VVC but also note potential failure to eradicate fungal load due to formation of thick, confluent biofilm layers on vaginal mucosa.

Although *L. rhamnosus* has been extensively studied for its probiotic activity against *C. albicans*, **Chapter 5** utilised RNA sequencing to assess the molecular interactions between *L. crispatus*, and *C. albicans* to examine the probiotic potential of a health-associated vaginal commensal. *L. crispatus* has been shown to inhibit *C. albicans* biofilm formation and promote stability of the healthy vaginal microbiome (Antonio et al., 1999, Verstraelen et al., 2009). Data from this experiment revealed, for the first time, a potential α -amino acid biosynthesis and amino acid starvation driven mechanism by which *L. crispatus* may out-compete *C. albicans* to re-establish a healthy vaginal microbiome. This process of up-regulating genes involved in amino acid biosynthesis such as *HIS5*, *ARG3* and *LEU42* whilst downregulating *CAR1* and *CAR2*, involved in breakdown, allows for an extracellular accumulation of amino acids which can be utilised for metabolism by *L. crispatus*. Additionally, it was noted that the two microbes did not begin to interact until 24 h of incubation, likely owed to building levels of secreted metabolites of *L. crispatus*, as shown previously (Ogunshe et al., 2011). This result means it may be beneficial for

daily addition of *L. crispatus* probiotics for the treatment of VVC to allow inhibitory effects to occur. Further research to elucidate the influence of secreted metabolites such as bacteriocins and H₂O₂, as well as other *Candida* and *Lactobacillus* species, are required to fully understand the observed antagonism between these microbes in the vaginal environment. A greater depth of knowledge would uncover the feasibility of *Lactobacillus* probiotics in the *in vivo* setting and potentially lead to the development of more appropriate therapies to recover a healthy vaginal microbiome during VVC.

Another important conclusion from **Chapter 5** was the ability of *L. crispatus* to reduce *C. albicans* composition within a polymicrobial biofilm model. This is the first report of *C. albicans* biofilm reduction by *L. crispatus* in an *in vitro* polymicrobial biofilm setting. A similar reduction has been shown previously using mono-species *C. albicans* biofilms (Rizzo et al., 2013, Wang et al., 2017, Li et al., 2019). Despite various studies investigating *C. albicans* biofilm reduction by probiotic *Lactobacillus* species, the mechanism of this reduction remains largely unknown. Pre-treatment of human epithelial cells with *L. crispatus* has been shown to maintain immune homeostasis by reducing inflammatory biomarker expression as well as *C. albicans* adhesion and virulence (Rizzo et al., 2013, Li et al., 2019). Further transcriptomic studies are required to fully elucidate the mechanisms governing recorded antagonism between different *Candida* and *Lactobacillus* species which could lead to the development of novel probiotic therapies or augment current therapies to treat VVC. Collectively, the data from **Chapter 5** exploits a potential mechanism by which *L. crispatus* strongly supports the use of *Lactobacillus* probiotics for the positive modulation of the vaginal microbiome during VVC.

6.5 Future work

Work from this thesis has identified key caveats in our knowledge pertaining to the formation of *Candida* biofilms on vaginal mucosa during RVVC and the interactions between *Candida* and *Lactobacillus*. Additionally, it provides a foundation for future studies to further investigate the presence of *Candida* biofilms during RVVC *in vivo*. Vaginal biopsies collected as part of large-scale clinical studies designed to visualise *Candida* biofilm formation should be conducted to conclude their importance in RVVC. Further, the microbial populations identified in this PhD study, and others, could be used to create an *in vitro* biofilm model representative of VVC which would allow for high-throughput assessment of novel therapeutics and compositional analyses. Finally, this study provides the foundation for a greater understanding of antagonism between *C. albicans* and *L. crispatus* through further investigation of the α -amino acid biosynthesis/starvation response identified here. Collectively, these findings could greatly improve our understanding of the microbial fluctuations leading to RVVC onset, identify the need for specific biofilm treatments for RVVC, and lead to the development of more appropriate therapeutics for women with recurrent, resistant episodes.

6.6 Concluding remarks

In conclusion, the work from this thesis has investigated the role of *Candida* biofilm formation, the influence of bacterial populations, and important interactions between *Candida* and *Lactobacillus* during RVVC through a range of microbiological, immunological and metagenomic approaches. It is important to note that, whilst most episodes of VVC can be treated with current azole therapies, high levels of recurrence remain. These debilitating recurrent cases greatly impact women's mental health and have a significant burden on the global economy. Until the controversy pertaining to *Candida* biofilm formation on vaginal mucosa and the efficacy of *Lactobacillus* probiotics is resolved, treatment options will remain limited to often unsuccessful, prolonged suppressive therapies.

The key findings of this PhD study are outlined below. A graphical illustration of important features identified by the clinical study in this thesis can be found in Figure 6.1.

- Clinical samples collected from patients with RVVC show evidence of *Candida* biofilm formation which could greatly impact clinical outcome.
- Specific health-associated *Lactobacillus* species including *L. crispatus* and *L. jensenii* are gradually replaced by *L. iners* during RVVC.
- *L. crispatus* utilises an α -amino acid biosynthesis/starvation driven mechanism to reduce *C. albicans* within an *in vitro* biofilm model which may allow it to re-establish a healthy microbiome.

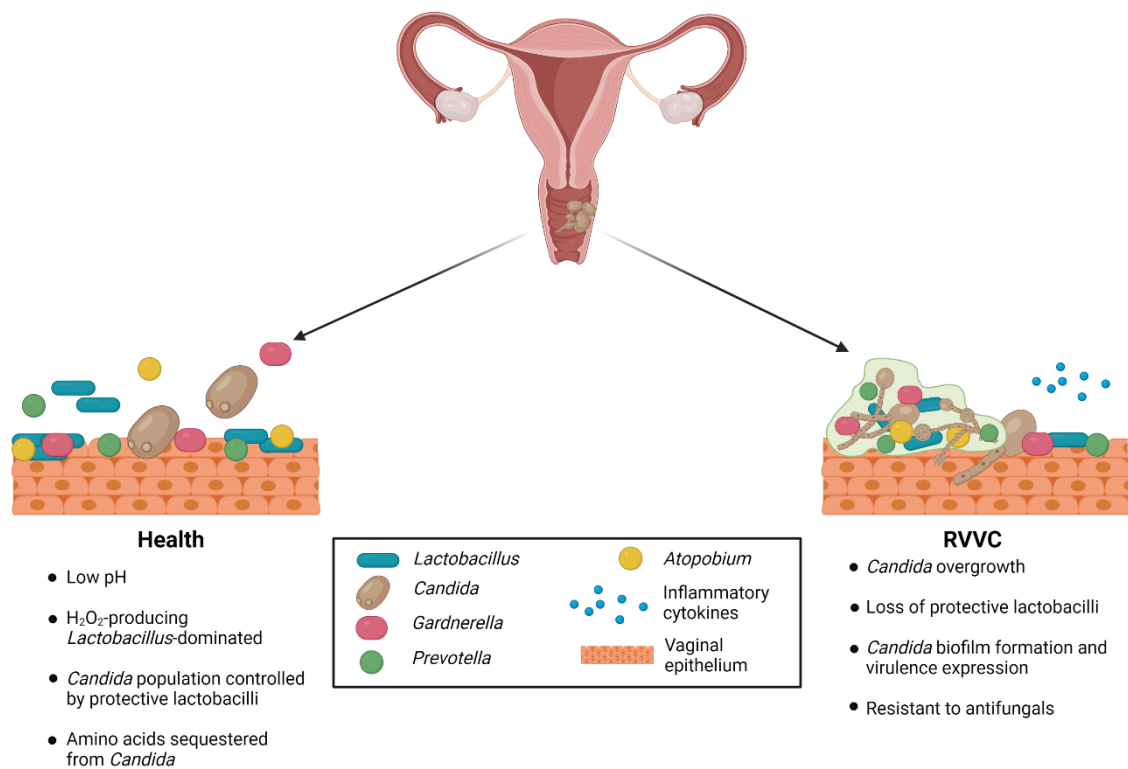


Figure 6.1: Graphical illustration of the key findings of this PhD study. Chapter 3 found an increased fungal load and expression of pro-inflammatory cytokines to be associated with RVVC. Further, data in this chapter displayed strong evidence of *Candida* biofilm formation during RVVC including biofilm-related gene expression, ability to form biofilms *in vitro* and visualisation of yeast aggregates in lavage fluid. Chapter 4 identified a *Lactobacillus*-dominated community with low levels of vaginal anaerobes in both cohorts, with a reduction of protective *Lactobacillus* species in RVVC. Data in Chapter 5 identified an α -amino acid driven mechanism by which *L. crispatus* can maintain health during colonisation with *C. albicans*, which is lost during disease. Created with Biorender.com.

7 Appendices

Appendix i: Patient information sheet (Health)

Participant Information Sheet (Health group)

An investigation of recurrent vaginal candidiasis (thrush)– how do microorganism communities contribute towards infections?

You are being invited to take part in a research study at the Sandyford Sexual Health Service. Contact details:

Dr Rebecca Metcalfe
Specialist Registrar, Sexual Health & HIV
Sandyford Sexual Health Service
2-6 Sandyford Place
Glasgow, G3 7NB
Tel:0141 211 8130

Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Contact us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. This is a joint study with NHS Greater Glasgow & Clyde and the School of Medicine at the University of Glasgow.

What is the purpose of the study?

To find out if communities consisting of bacteria and fungi, also known as biofilms, are present in the vagina, and if they contribute to recurrent vaginal thrush. This will be assessed using standard laboratory culture techniques and cutting-edge molecular biology methods.

Why have I been asked to take part?

You have been chosen as you are having an intrauterine device (coil) inserted and will serve as a healthy control for this study. Approximately 30% of healthy women have fungal communities without active thrush; therefore if it is detected in your sample there is nothing to worry about. If your sample does not contain the fungal community, no further analysis will be carried out on it. We aim to recruit 60 patients with thrush and 60 healthy controls from women who have a coil inserted into this study.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you decide not to take part, it will not affect the healthcare that you receive in any way.

What will happen if I take part?

After consenting to take part, you will get ready to be examined as normal. Before the usual examination, you will have one swab sample taken from your vagina and a vaginal wash will be carried out; this means the doctor will put 5 ml (a teaspoon) of sterile water around the vagina to 'wash' the area and then remove it all. These tests will take about 2 minutes to complete. This sampling process will not cause any further discomfort than routine sampling. This will be done before the intrauterine device insertion. These samples are purely for research purposes and do not support your care. If during examination the clinician detects an infection, you will be informed and given appropriate treatment.

The samples that are taken will be analysed anonymously at the School of Medicine, University of Glasgow – there will be no way of identifying you or any individuals from the samples.

What are the possible benefits of taking part?

You may not get a direct benefit from taking part in this study, however it will help us to find out whether fungal communities are related to recurrent vaginal thrush. This may benefit patients in the future with better management of this condition.

What are the possible disadvantages and risks of taking part?

There should be no disadvantages or risks from taking part. The tests are safe and should not cause you any more discomfort than the routine examination you will be having today.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence, then you may have grounds for a legal action for compensation against NHS Greater Glasgow & Clyde but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

What happens when the study is finished?

The findings will be published in scientific journals and presented at scientific conferences. You will not be identified in any report or publication. If you would like to know the outcome of the results from your tests, please contact Dr Rebecca Metcalfe on the contact details below.

Will my taking part in the study be kept confidential?

All the information we collect during the course of the research will be kept confidential and there are strict laws, which safeguard your privacy at every stage.

Appendices

Your name will be removed from the data and you will be given a unique number so that you cannot be recognised from the data.

Who has reviewed the study?

This study has been reviewed by NHS Greater Glasgow & Clyde Research and Development Management Office. In addition, all research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests.

If you have any further questions about the study please contact:

Dr Rebecca Metcalfe
Specialist Registrar Sexual Health & HIV
Sandyford Sexual Health Service
2-6 Sandyford Place
Glasgow, G3 7NB
Tel:0141 211 8130

If you wish to make a complaint at any stage please contact:

Complaints Team
Glasgow City CHP – North West Sector J B Russell House
Gartnavel Royal Hospital
1055 Great Western Road
Glasgow G12 0XH
0141 211 0370

Thank you for reading this information and considering taking part in this research study.

Appendix ii: Patient information sheet (RVVC)

Participant Information Sheet (RVVC group)

An investigation of recurrent vaginal candidiasis (thrush)– how do microorganism communities contribute towards infections?

You are being invited to take part in a research study at the Sandyford Sexual Health Service. Contact details:

Dr Rebecca Metcalfe
Specialist Registrar, Sexual Health & HIV
Sandyford Sexual Health Service
2-6 Sandyford Place
Glasgow, G3 7NB
Tel:0141 211 8130

Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Contact us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. This is a joint study with NHS Greater Glasgow & Clyde and the School of Medicine at the University of Glasgow.

What is the purpose of the study?

To find out if communities consisting of bacteria and yeast, also known as biofilms, are present in the vagina, and if they contribute to recurrent vaginal thrush. This will be assessed using standard laboratory culture techniques and cutting-edge molecular biology methods.

Why have I been asked to take part?

You have been chosen as you currently suffer from vaginal thrush. We aim to recruit 60 patients with thrush and 60 healthy controls from women who have a coil inserted into this study.

We aim to include 120 patients in this study.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving

a reason. If you decide not to take part, it will not affect the healthcare that you receive in any way.

What will happen if I take part?

After consenting to take part, you will get ready to be examined as normal. Before the usual examination, you will have one swab sample taken from your vagina and a vaginal wash will be carried out; this means the doctor will put 5 ml (a teaspoon) of sterile water around the vagina to 'wash' the area and then remove it all. The tests will take about 2 minutes to complete. This sampling process will not cause any further discomfort than routine sampling. This will be done after any other samples the doctor wants to take. These samples are purely for research purposes and do not support your care.

The samples that are taken will be analysed anonymously at the School of Medicine, University of Glasgow – there will be no way of identifying you or any individuals from the samples. In addition, if you are having a routine swab taken, it will be analysed by NHS GG&C as usual and the results shared with the research group at the University of Glasgow. Again, at no point will you be identifiable by these samples.

What are the possible benefits of taking part?

You may not get a direct benefit from taking part in this study, however it will help us to find out whether fungal communities are related to recurrent vaginal thrush. This may benefit patients in the future with better management of this condition.

What are the possible disadvantages and risks of taking part?

There should be no disadvantages or risks from taking part. The tests are safe and should not cause you any more discomfort than the routine examination you will be having today.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence, then you may have grounds for a legal action for compensation against NHS Greater Glasgow & Clyde but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

What happens when the study is finished?

The findings will be published in scientific journals and presented at scientific conferences. You will not be identified in any report or publication. If you would like to know the outcome of your personal results please contact Dr Rebecca Metcalfe on the contact details below.

Will my taking part in the study be kept confidential?

All the information we collect during the course of the research will be kept confidential and there are strict laws, which safeguard your privacy at every stage. Your name will be removed from the data and you will be given a unique number so that you cannot be recognised from the data.

Who has reviewed the study?

This study has been reviewed by NHS Greater Glasgow & Clyde Research and Development Management Office. In addition, all research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests.

If you have any further questions about the study please contact:

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Complaints Team
Glasgow City CHP – North West Sector J B Russell House
Gartnavel Royal Hospital
1055 Great Western Road
Glasgow G12 0XH
0141 211 0370

Thank you for reading this information and considering taking part in
this research study.

Appendix iii: Patient consent form

CONSENT FORM

Title of Project: An investigation of recurrent vaginal candidiasis (thrush)– how do microorganism communities contribute towards infections?

Name of Researcher:

Please initial box -

I confirm that I have read and understand the patient information sheet dated Version 06, 08/02/2017) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

I understand that my participation is voluntary and that **I am free to refuse sampling or withdraw from the study at any time** without giving any reason and without my medical care or legal rights being affected. ☐

I understand that relevant sections of my medical notes and data collected during the study may be looked at by regulatory authorities or from the NHS organisation, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

I agree to my data being transferred in an anonymised form from NHS Greater Glasgow & Clyde to the School of Medicine (University of Glasgow) for analysis. This includes the identification of the organism within my samples. ☐

I agree to take part in the above study. ☐

Name of Patient Date.....

Signature

Name of Person taking consent..... Date.....

Signature

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Appendix iv: Patient questionnaire

PATIENT QUESTIONNAIRE

Title of Project: An investigation of recurrent vaginal candidiasis (thrush)— how do microorganism communities contribute towards infections?

Please answer the following questions to the best of your knowledge -

1. How many times have you suffered from vaginal thrush within the last year?

.....

2. What products have you used to help treat this infection e.g. Canestan?

.....

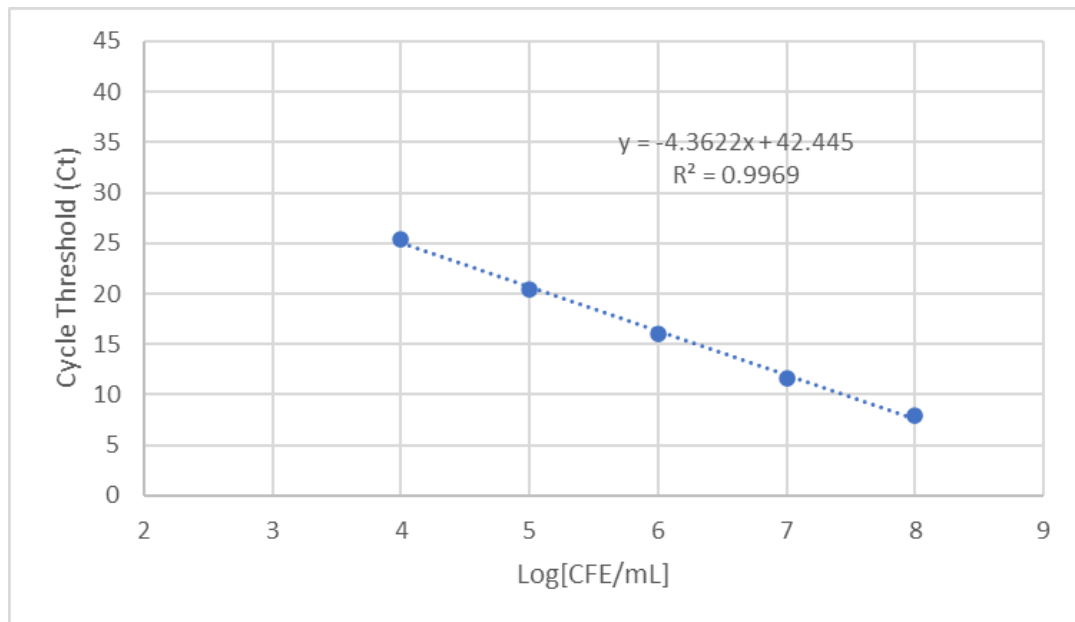
3. When was the last time you treated this infection?

.....

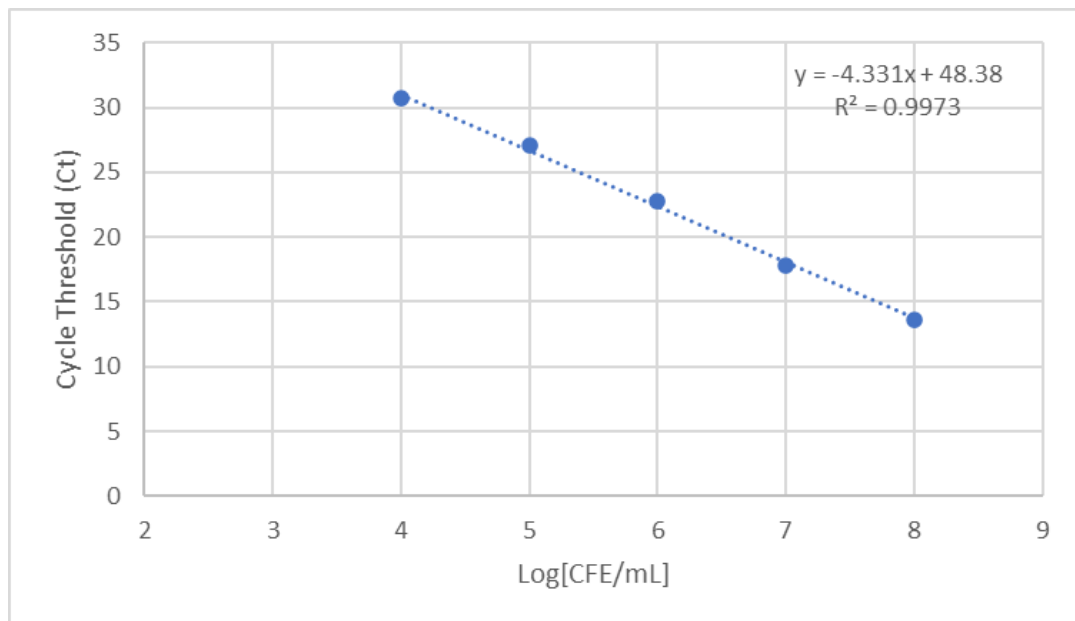
4. Are you currently on any contraception? If so, what kind?

.....

Appendix v: *C. albicans* SC5314 standard curve for qPCR calculations



Appendix vi: *E. coli* K12 standard curve for qPCR calculations



Appendix vii: Recurrent Vulvovaginal Candidiasis: a Dynamic Interkingdom Biofilm Disease of *Candida* and *Lactobacillus*

RESEARCH ARTICLE



Recurrent Vulvovaginal Candidiasis: a Dynamic Interkingdom Biofilm Disease of *Candida* and *Lactobacillus*

Emily McCloud,^a Christopher Delaney,^a Leighann Sherry,^b Ryan Kean,^c Shanice Williams,^a Rebecca Metcalfe,^d Rachael Thomas,^d Riina Richardson,^e Konstantinos Gerasimidis,^a Christopher J. Nile,^f Craig Williams,^a Gordon Ramage^a

^aSchool of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, Glasgow, UK ^bSchool of Life Sciences, Glasgow, UK ^cDepartment of Biological and Biomedical Sciences, School of Health and Life Sciences, Glasgow Caledonian University, Glasgow, UK ^dSandyford Sexual Health Service, NHS Greater Glasgow and Clyde, Glasgow, UK ^eDivision of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK ^fSchool of Dental Sciences, University of Newcastle, Newcastle upon Tyne, UK

Emily McCloud and Christopher Delaney contributed equally to this work. Author order was determined by drawing straws.

ABSTRACT Despite the strikingly high worldwide prevalence of vulvovaginal candidiasis (VVC), treatment options for recurrent VVC (RVVC) remain limited, with many women experiencing failed clinical treatment with frontline azoles. Further, the cause of onset and recurrence of disease is largely unknown, with few studies identifying potential mechanisms of treatment failure. This study aimed to assess a panel of clinical samples from healthy women and those with RVVC to investigate the influence of *Candida*, the vaginal microbiome, and how their interaction influences disease pathology. 16S rRNA sequencing characterized disease by a reduction in specific health-associated *Lactobacillus*

Citation McCloud E, Delaney C, Sherry L, Kean R, Williams S, Metcalfe R, Thomas R, Richardson R, Gerasimidis K, Nile CJ, Williams C, Ramage G. 2021. Recurrent vulvovaginal candidiasis: a dynamic interkingdom biofilm disease of *Candida* and *Lactobacillus*. *mSystems* 6: e00622-21. <https://doi.org/10.1128/mSystems.00622-21>.

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Address correspondence to Gordon Ramage, gordon.ramage@glasgow.ac.uk.

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species, such as *Lactobacillus crispatus*, coupled with an increase in *Lactobacillus iners*. *In vitro* analysis showed that *Candida albicans* clinical isolates are capable of heterogeneous biofilm formation, and we found the presence of hyphae and *C. albicans* aggregates in vaginal lavage fluid. Additionally, the ability of *Lactobacillus* to inhibit *C. albicans* biofilm formation and biofilm-related gene expression was demonstrated. Using RNA sequencing technology, we were able to identify a possible mechanism by which

L. crispatus may contribute to re-establishing a healthy vaginal environment through amino acid acquisition from *C. albicans*. This study highlights the potential formation and impact of *Candida* biofilms in RVVC. Additionally, it suggests that RVVC is not entirely due to an arbitrary switch in *C. albicans* from commensal to pathogen and that understanding interactions between this yeast and vaginal *Lactobacillus* species may be crucial to elucidating the cause of RVVC and developing appropriate therapies.

IMPORTANCE RVVC is a significant burden, both economically and for women's health, but its prevalence is poorly documented globally due to the levels of self-treatment. Identifying triggers for development and recurrence of VVC and the pathogenesis of the microbes involved could considerably improve prevention and treatment options for women with recurrent, azole-resistant cases. This study therefore aimed to examine the interkingdom dynamics from healthy women and those with RVVC using next-generation sequencing techniques and to further investigate the molecular interactions between *C. albicans* and *L. crispatus* in a relevant biofilm coculture system.

KEYWORDS antifungal resistance, biofilm, *Candida*, clinical, interkingdom,

Appendices

Lactobacillus, microbiome, vulvovaginal candidiasis

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Fungal infections are becoming increasingly recognized as a substantial health burden on the global population. Over 1 billion people are estimated to suffer from fungal infections each year, resulting in over 1.5 million deaths (1). These infections are commonly associated with mucosal sites, such as the vagina, gut, and oral cavity. Vaginitis is estimated to account for up to 7% of all visits to gynecologists and for up to 10 million general practitioner (GP) appointments annually (2). Vulvovaginal candidiasis (VVC) is not a reportable disease and is often self-treated with over-the-counter antifungal agents; therefore, its exact prevalence and distribution are impossible to determine. VVC is reported as the second most common cause of vaginitis, and it is estimated that 75% of women will suffer from VVC in their childbearing years, with up to 140 million of these women developing recurrent VVC (RVVC), defined as ≥ 4 cases within 1 year (3, 4). These recurrent cases are debilitating, impact quality of life, and are associated with psychological stress, pruritis, and discomfort (5, 6). VVC is associated with various risk factors, including the use of antibiotics and contraceptives, new sexual partners, and allergic responses to *Candida* antigens; however, no distinguishable cause has been identified (7, 8).

The biofilm-forming yeast *Candida albicans* is reported as the predominant pathogen responsible for up to 90% of VVC infections (4). *C. albicans* is commonly isolated from the vagina as an asymptomatic commensal with a carriage rate of up to 33% in premenopausal women (9). Other non-*albicans* *Candida* (NAC) species, most commonly *Candida glabrata*, *C. parapsilosis*, *C. dubliniensis* and *C. krusei*, account for 10 to 20% of VVC infections and are associated with complicated VVC, which exhibits less severe symptoms than *C. albicans* VVC but higher recurrence rates (10, 11). *C. albicans* has been shown to form biofilms on vaginal mucosa both *ex vivo* and *in vivo* by identification of significant fungal load, biofilm architecture, and extracellular matrix using confocal and electron microscopy (12). This is a possible contributing factor to failed clinical treatment resulting in persistent and recurrent VVC. Despite the identification of *Candida* biofilms on vaginal mucosa, the predicted therapeutic challenge this presents in VVC is still disputed. Other studies challenge the hypothesis of vaginal biofilms and instead suggest other factors, such as germ tube formation and

polymicro-bial tissue invasion, as more critical features of VVC (13, 14).

The vaginal microbiome is somewhat unique in that a healthy microbiome is associated with a less diverse community of microbes, predominantly lactobacilli such as *Lactobacillus crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii* (15). Dysbiosis of the vaginal microbiome leads to an increase in species diversity, with fewer lactobacilli and greater numbers of pathogenic anaerobes, such as *Gardnerella* and *Prevotella* (16, 17). In healthy women, lactic acid bacteria (LAB) are thought to be responsible for maintaining a homeostatic microbiome by inhibiting growth and adhesion of other microbes via the production of secreted metabolites, such as lactic acid, biosurfactants, bacteriocins, and H₂O₂. *L. crispatus*, a potent producer of both D- and L- isomers of lactic acid as well as H₂O₂, is a prevalent commensal of the healthy vaginal environment in various microbiome studies (18, 19). Additionally, it is known to secrete the L-lactic acid isomer that has been extensively studied for its ability to lower vaginal pH, elicit anti-inflammatory responses, and inhibit microbial colonization (20, 21).

A single dose of oral fluconazole is sufficient to treat sporadic *C. albicans* VVC in 80 to 90% of cases (22). Treatment of VVC caused by NAC is more complicated, requiring prolonged suppressive azole therapies, and is often unsuccessful. The efficacy of therapeutics such as amphotericin B and boric acid has been assessed for the treatment of RVVC. Both drugs when delivered intravaginally for 14 to 21 days were found to be effective in around 70% of patients (23, 24). *Candida* species, predominantly *C. albicans*, are known to form thick tenacious biofilms that dramatically increase tolerance to antifungal drugs commonly used in the treatment of VVC, such as fluconazole, miconazole, and flucytosine (25). Indeed, sessile cells have been shown to tolerate antifungal concentrations 1,000-fold greater than their planktonic counterparts (26). Therefore, an alternative therapeutic strategy for RVVC may be through microbiome replacement therapy in the form of

Lactobacillus probiotics (27). Probiotic therapy involves the administration of live microorganisms, which directly results in a health benefit for the patient (28). Due to the diversity of lactobacilli within the vaginal microbiome, it is difficult to estimate which species would be most important to replace with probiotic therapy. One study evaluated *Lactobacillus plantarum* P17630 combined with the standard treatment of clotrimazole for 3 days and concluded a potential resolution of vaginal discomfort (29). The probiotic potential of secreted metabolites of *Lactobacillus* against *C. albicans* has been summarized, identifying gaps in our knowledge of fungal-*Lactobacillus* interactions that could lead to improved treatment options (30–33). Currently, no probiotic therapies have been approved for the treatment of VVC/RVVC.

Clearly, RVVC is a significant burden, both economically and for women's health, and its prevalence is poorly documented globally due to the levels of self-treatment. Identifying triggers for development and recurrence of VVC and the pathogenesis of the microbes involved could considerably improve prevention and treatment options for women with recurrent, azole-resistant cases. This study therefore aimed to examine the interkingdom dynamics in healthy women and those with RVVC using next-generation sequencing techniques and to further investigate the molecular interactions between *C. albicans* and *L. crispatus* in a relevant biofilm coculture system.

RESULTS

Clinical and microbiological assessment of patient samples. The mechanisms behind the shift in candidal species from commensal to pathogenic yeast seen in RVVC onset remain poorly understood. The cause of infection is likely multifactorial, and here, we aimed to assess whether fungal or bacterial load may influence disease by using samples collected from healthy individuals ($n = 60$) and women with RVVC ($n = 40$). Clinical assessment was used to confirm RVVC diagnosis, which was further validated by significantly increased interleukin 8 (IL-8) expression levels ($P = 0.0001$) (Table S1). Patient demographic data collected through questionnaires found that patients had suffered from RVVC for an average of 8 months, with an average of 1 month since they last received treatment. Although higher numbers of women with RVVC had culturable *Candida* than healthy women, quantitative PCR

(qPCR) analysis revealed *Candida* to be detectable in all but 2 patients.

First, the levels of *Candida* present in the clinical samples were quantified by determining numbers of CFU per milliliter, in addition to total yeast and bacterial DNA quantified by qPCR colony-forming equivalents (CFE) per milliliter (Fig. 1). Quantities of yeast CFU/ml showed a significant increase in *Candida* load in samples, from approximately 2×10^3 CFU/ml in healthy patients to 2.3×10^4 CFU/ml in RVVC patients ($P = 0.0079$) (Fig. 1a). Similarly, when assessed by qPCR, a significant increase of around 1.3-log was observed in patients with RVVC, to 3.8×10^5 CFE/ml ($P = 0.024$), as shown in Fig. 1b. These data suggest that increased levels of *Candida* could be a contributing factor to disease pathology or an indicator for VVC onset and subsequent recurrence. Additionally, *Candida* load was observed with respect to patient metadata. Although a slight reduction of between 0.5 and 1 log was observed in patients with disease for over 7 months, this was not significant (data not shown). An increase in fungal load from 1×10^4 to 5×10^4 CFU/ml was observed in women who had received treatment for RVVC longer than 1 month prior to sample collection ($P = 0.021$) (Fig. S2). Bacterial load was found to be comparable at $\approx 8 \times 10^7$ CFE/ml between healthy women and those with RVVC ($P = 0.779$) (Fig. 1c). Finally, correlation between bacterial and fungal load found significantly higher levels of *Candida* DNA per CFE/ml of bacterial DNA present in RVVC compared with healthy controls ($P = 0.0001$) (Fig. 1d).

Taxonomic classification of health and RVVC. To determine the bacterial taxa present in healthy women and those with RVVC, we performed 16S rRNA sequencing on DNA extracted from swab samples (Fig. 2). At the genus level, a *Lactobacillus*-dominated environment, accounting for up to 75% of the microbiome, was observed in both cohorts, with similar levels of diversity and the vaginal anaerobes *Gardnerella* and *Prevotella* (Fig. 2a and b). *Atopobium* was found to be slightly higher in healthy patients

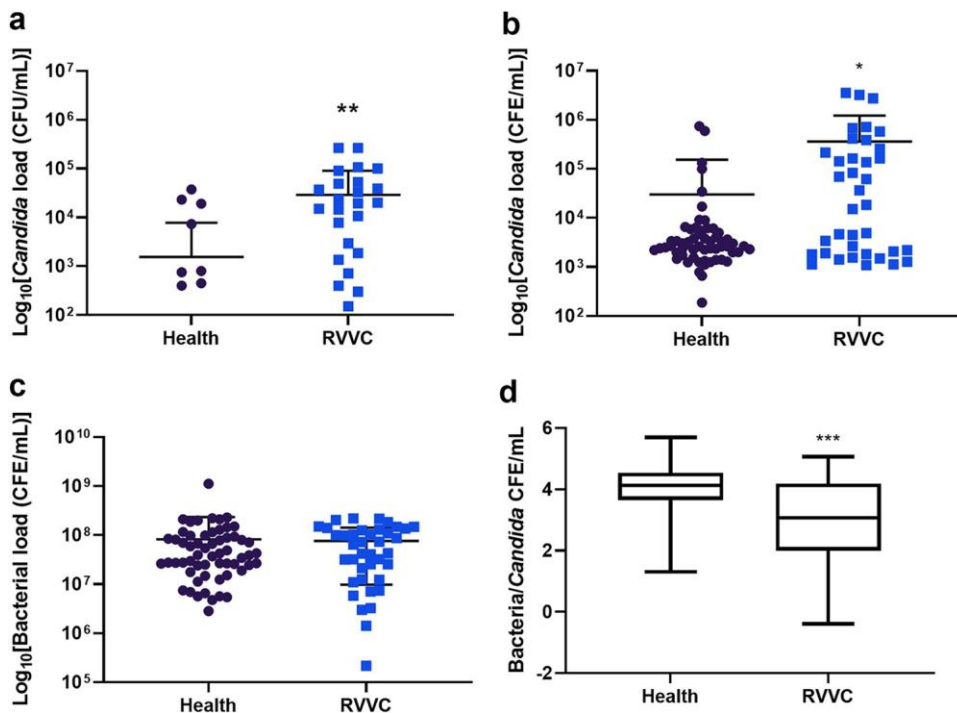


FIG 1 Fungal burden is elevated in women with RVVC while bacterial load remains unchanged. To assess fungal load, patient lavage was incubated on *Candida* Chromogenic agar plates and colonies counted after 48h (a). For calculation of CFE/ml, the ITS region of *Candida* was amplified using genus-specific primers (b). Levels of bacteria DNA were also assessed molecularly by amplification of the 16S rRNA region (c). Correlation between bacterial and fungal burden was also observed (d). Data are means \pm standard deviations (SD). Statistical significance was calculated using unpaired *t* tests with Welch's correction, as data did not share equal standard deviations (*, *P* \geq 0.05; **, *P* \leq 0.01).

and *Bifidobacterium* slightly lower. A limitation of our microbiome analysis was the inability to distinguish between some *Lactobacillus* species (including *Lactobacillus acidophilus*, *L. casei*, and *L. gasseri*). For this reason, these species are grouped as “*Lactobacillus* spp., including *L. crispatus*.” However, sequencing of a subset of samples using the Oxford Nanopore Technologies MinION sequencer confirmed accuracy to the species level (Fig. S3) (34). When viewed at the species level, although not significant following *P* value correction, subtle differences in bacterial taxa were observed between healthy controls and RVVC, particularly among *Lactobacillus* species (Fig. 3). Most notable was the reduction in levels of specific *Lactobacillus* species. This included

L. jensenii and to a greater extent *L. crispatus*, which fell from 44% in healthy patients to 30% in those with RVVC (Fig. 3a and b). Interestingly, this reduction was coupled with an increase in *L. iners* from just 19% in healthy samples to 40% in RVVC. Additionally, when predicted using random forest plots, levels of *L. iners* were sug-

gested to be the most distinct between samples from healthy women and those from women with RVVC (Fig. 3c).

To investigate the influence of bacterial taxa further, microbial populations were observed with respect to patient metadata (Fig. 4). In patients with culturable *Candida* compared with those who were culture negative, a reduction in *Lactobacillus* species including *L. crispatus* from 44% to 29%, coupled with an increase in *L. iners* from 23% to 35%, was observed, similar to the RVVC profile (Fig. 4a). Further, *L. iners* was predicted to be the second most likely organism to define presence or absence of *Candida* using random forest analysis to identify important features (Fig. 4c). When observed with respect to the length of time patients had had RVVC, an intermediate profile between health and disease was observed in patients with disease for less than 6 months, showing a slight reduction in *L. crispatus* from 45% to 40% (Fig. 4c). Conversely, patients with disease for between 6 and 12 months had a profile similar to that seen in RVVC, with increased levels

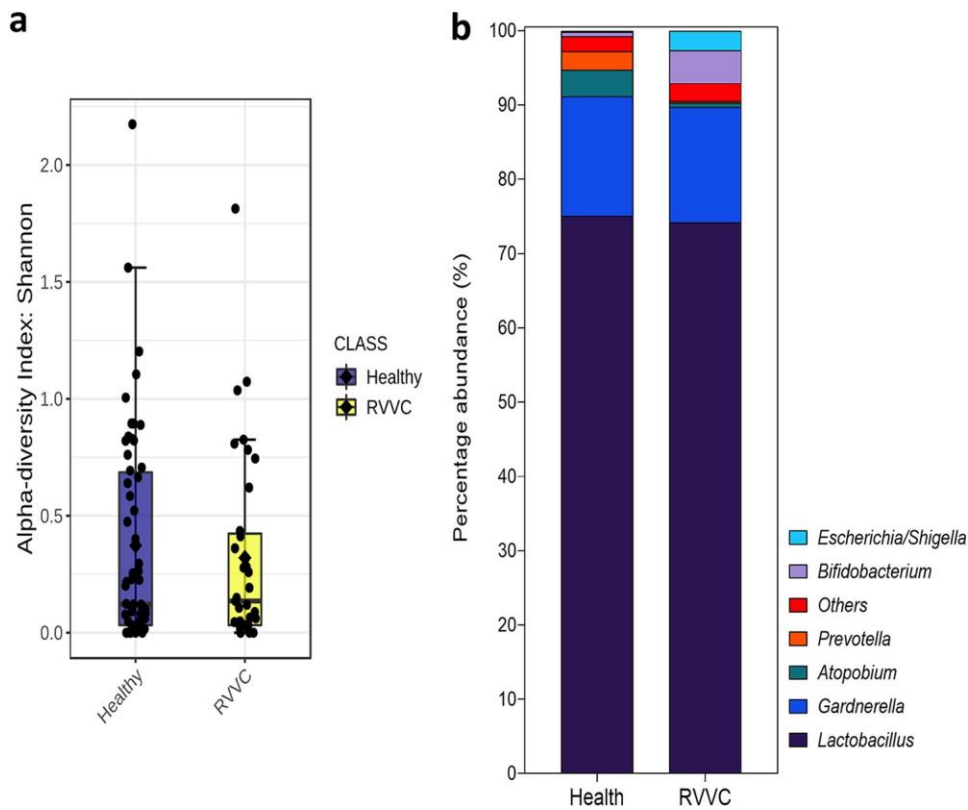


FIG 2 Bacterial genera present in healthy women and those with RVVC. DNA extracted from swab samples was used for 16S rRNA Illumina sequencing ($n=100$). Bacterial diversity measured by Shannon index (a) and genus-level taxa identification and percentage abundance of microbial populations present (b) in health and RVVC.

of *L. iners* from 19% to 44% (Fig. 4d). Additionally, bacterial communities present were observed with respect to contraceptives used at the time of sampling (Fig. S4). At the species level, a notable similarity in the composition of healthy patients and those using hormonal contraception, in contrast to patients using a contraceptive device or no contraception, was observed. The most important feature identified at the species level was predicted to be *Atopobium vaginae*. It is predicted that this organism is present in higher numbers in healthy patients and those using hormonal contraceptives and lower numbers in patients using either no contraception or contraceptive devices. Additionally, a contrasting pattern is shown for *L. iners*, which is predicted to be highest in patients using contraceptive devices. The microbiome was then observed with respect to how recently patients had received antifungal intervention to treat RVVC (Fig. S5). A gradual reduction in *Lactobacillus* species, including *L. crispatus*, was observed, from 44% in healthy samples to 34% and 23% in patients treated within a month and more than a month before sampling, respectively.

Patients treated more than 1 month before sampling were predicted to have an intermediate profile between those of healthy women and women treated for RVVC more recently.

Finally, correlation between the vaginal microbiome and *Candida* load (CFE/ml) was observed with respect to patient metadata (Fig. S6). *Megasphaera*, often associated with vaginal dysbiosis, was found to be significantly positively correlated with increased fungal load (P , 0.05) (Fig. S6). Although not significant, genera such as *Dialister*, *Bacteroides*, and *Shuttleworthia* were correlated with higher fungal loads in RVVC. Further, *Lactobacillus* was significantly negatively correlated with *Candida* in healthy samples, and to a lesser extent in RVVC (P , 0.05). Although none were found to be significant, correlations observed with respect to recent treatment identified slight negative correlations between genera such as *Streptococcus*, *Gardnerella*, and *Lactobacillus* with increasing time since last RVVC intervention. Conversely, genera

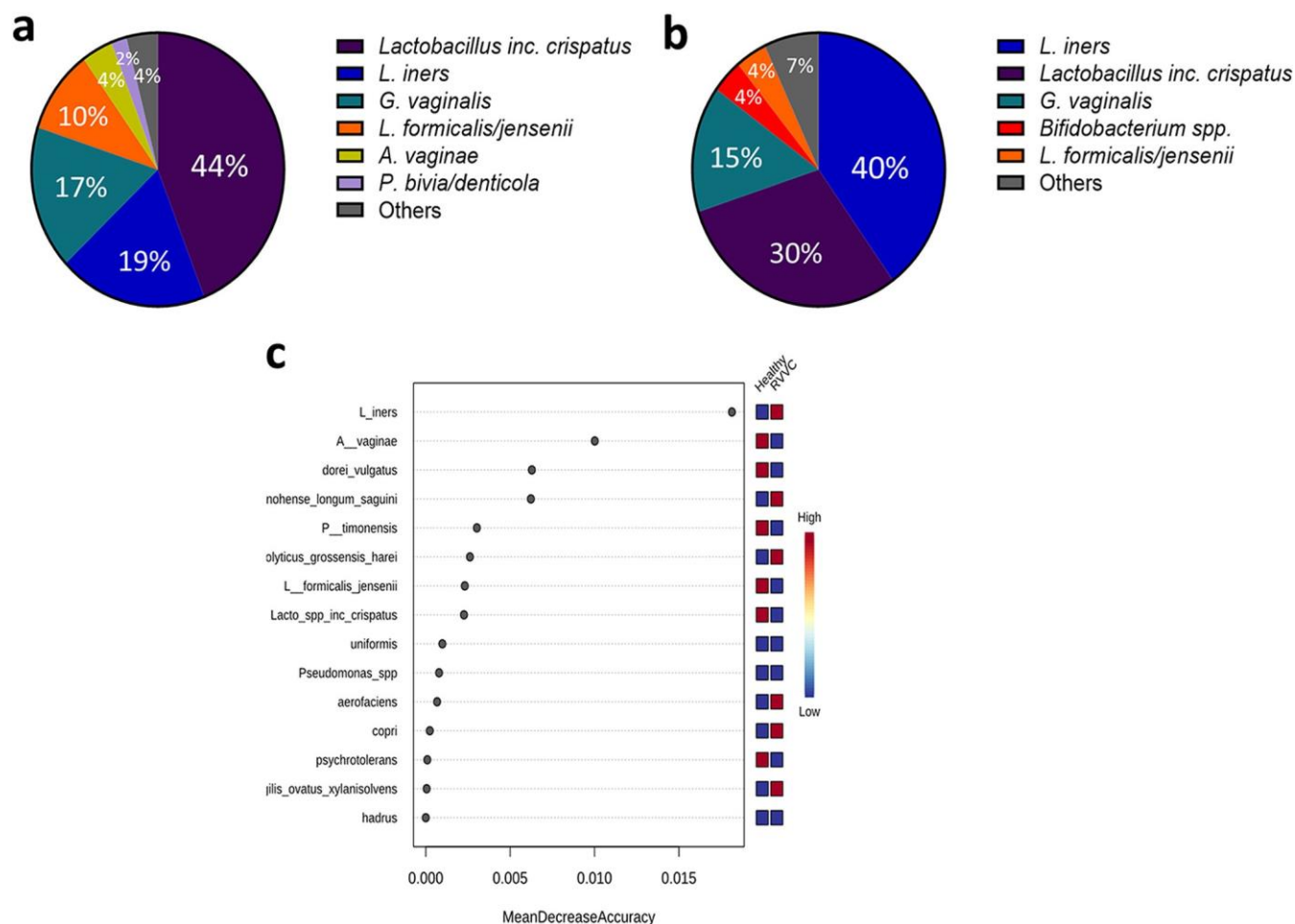


FIG 3 Hydrogen peroxide-producing lactobacillus strains are reduced during RVVC infection, resulting in an *L. iners*-dominated microbiome. Species-level identification of bacterial taxa present in healthy women (a) and those with RVVC (b). Random forest plot showing the most distinct species-level taxa present between healthy women and those with RVVC (c).

such as *Prevotella*, *Morganella*, and *Sneathia* were found to be positively correlated with increasing time since treatment. *Streptococcus*, *Prevotella*, and *Clostridium* were found to be positively correlated with increased RVVC episodes. However, genera such as *Shuttleworthia*, *Lactobacillus*, and *Atopobium* were found to be negatively correlated with increasing RVVC episodes.

Influence of *Candida* biofilm formation in RVVC. *Candida* clinical isolates were obtained from lavage samples by culture on *Candida* chromogenic agar. Following incubation, a total of 33 isolates were obtained, 9 from healthy individuals and 24 from patients with RVVC. Isolate identification was confirmed for all isolates using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) (Fig. 5a). Consistent with previous RVVC studies, *C. albicans* was found to account for 73% of the *Candida* species isolated within our patient subset. Other NAC species, more commonly associated with RVVC, accounted for the remaining 27%. Following identification, biofilm forming capabilities of clinical isolates was assessed (Fig. 5b). *C.*

albicans was capable of forming dense biofilms with clear heterogeneity between isolates. Isolates were grouped as low biofilm formers (LBF) if the absorbance reading of their total biomass fell below the first quartile (≤ 0.185). Similarly, high biofilm formers (HBF) were observed when total biomass was above the third quartile (≥ 0.854). Intermediate biofilm formers (IBF) had biomass readings between these values. Minimal biomass was observed in NAC species. Additionally, when lavage fluid from an RVVC patient with a

C. albicans isolate capable of dense biofilm formation was viewed microscopically,

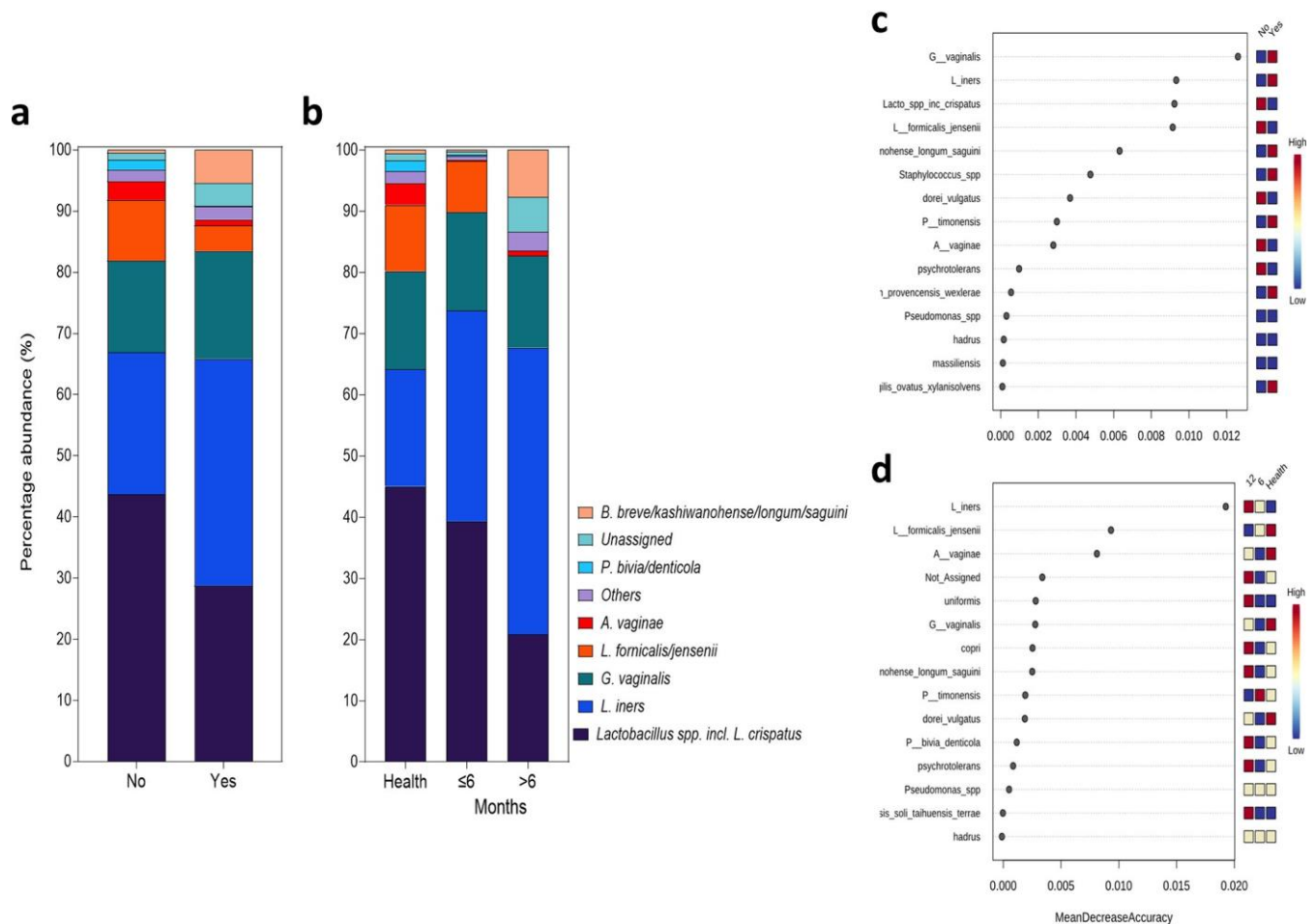


FIG 4 Species-level taxon abundance relative to patient metadata. Species-level bacterial taxa based on presence/absence of *Candida* (a) and length of time with disease (b). Random forest plots showing distinct bacterial taxa present in each analysis (c and d).

hyphal forms and bacterium-yeast aggregates could be clearly observed (Fig. 5c). To further investigate and validate that *Candida* biofilms are a defined clinical entity, expression levels of key biofilm-related genes were measured from patients' vaginal lavage fluid. These data show detectable levels of expression of genes involved in hyphal morphogenesis, biofilm formation, and pathogenesis in *Candida* isolates present in patients with RVVC, including *HWP1*, *ECE1*, *ALS3*, and *SAP* (Table S2).

Antagonism of *Lactobacillus* and *C. albicans*. Next, to assess the interactions between *Lactobacillus* species and *C. albicans*, 7 *Lactobacillus* species were selected and their effects on *C. albicans* biofilm formation in coculture observed (Fig. 6). When grown together for 24 h, a reduction in overall biomass was observed in all *Lactobacillus* species from an absorbance reading of 3.0 to approximately 2.5. This reduction was particularly prominent in *Lactobacillus rhamnosus*,

where biomass was reduced to an absorbance value of 2.0 (Fig. 6a). However, when *C. albicans* was allowed to form a biofilm before addition of *Lactobacillus*, this effect was less pronounced and in some cases absent (Fig. 6b). To further analyze the biomass reduction, *C. albicans* biofilm-related gene expression was assessed following coculture with *L. rhamnosus* and *L. iners*. *L. rhamnosus* is often studied for its probiotic effect against *C. albicans*. Although *L. iners* is a vaginal commensal, it has not been found to inhibit fungal growth or biofilm formation. The two organisms differentially interacted with *C. albicans*, with *L. rhamnosus* downregulating all biofilm-related gene expression after incubation for 20 h and 24 h (Fig. 6c). Gene expression was reduced by ≥ 4 -fold for *HWP1* and between 0.5- and 1-fold for *ALS3* and *ECE1* in both growth conditions. *L. iners* downregulated expression of *ECE1* and, to a

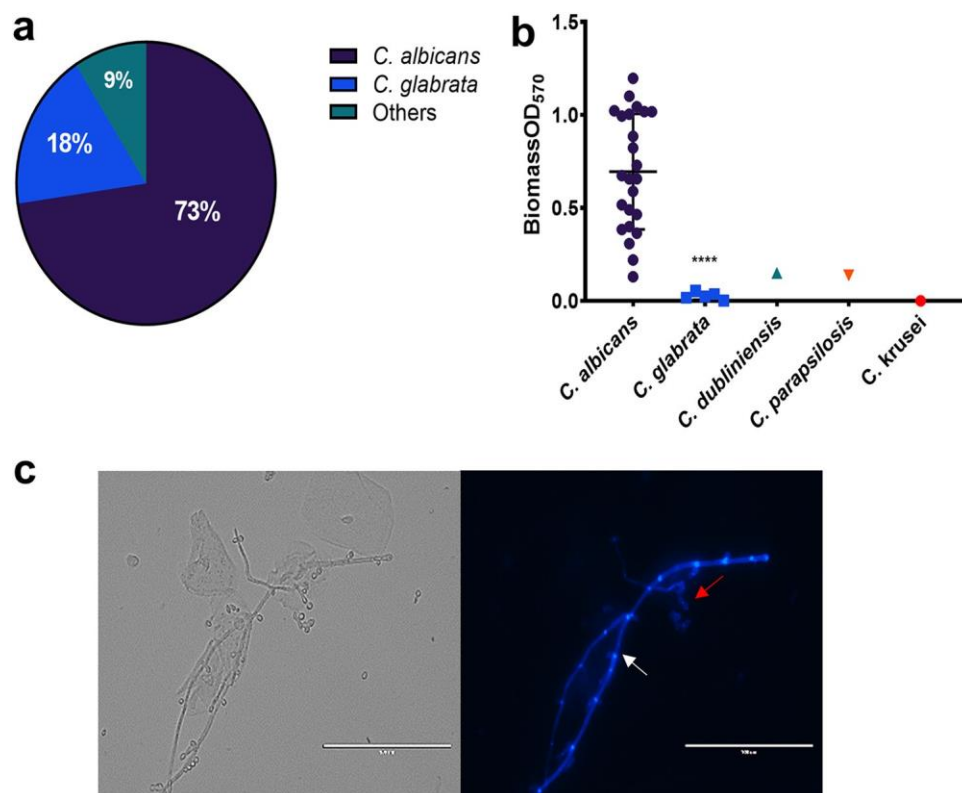


FIG 5 Vaginal *Candida* isolates from patients with RVVC are capable of heterogeneous biofilm formation. A total of 33 *Candida* clinical isolates were isolated from lavage samples. MALDI-TOF was used to identify each isolate and assess species distribution ("Others" comprises 1 isolate each of *C. dubliniensis*, *C. parapsilosis*, and *C. krusei*) (a). *Candida* isolates from healthy women ($n = 9$) and those with RVVC ($n = 24$) were assessed for biofilm-forming capabilities by crystal violet staining (b). Vaginal lavage was stained with calcofluor white (CFW) for 1 h at 37°C before imaging (c). Images are representative of *C. albicans* aggregates and hyphae observed in lavage fluid from a patient with an HBF isolate. White arrows represent pseudohyphal/hyphal formation, and red arrows depict cell aggregates. Data are means \pm SD. Statistical analysis was performed using unpaired t-tests (****, $P < 0.0001$).

lesser extent, *HWP1* after incubation for 24 h. However, when added to a preexisting *C. albicans* biofilm, *L. iners* resulted in upregulation of *ECE1* and *ALS3*.

To further understand how antagonistic features of *Lactobacillus* impact *C. albicans*, we utilized transcriptome sequencing (RNA-Seq) of *C. albicans* following coculture with *L. crispatus*. *L. crispatus* was selected for RNA sequencing analysis, as opposed to *L. rhamnosus*, as it is not a commensal of the vaginal environment and is capable of only transient colonization. Further, *L. crispatus* is one of the most prevalent *Lactobacillus* species in the vaginal microbiome, and its antagonism toward *C. albicans* has been demonstrated in previous studies (31–33, 35). Initially, multivariate analysis by principal-component analysis (PCA) was unable to identify distinct clusters of gene expression in early dual-species biofilms (Fig. S8). Subsequently, there was no significant up- or downregulation in early dual-species biofilm transcripts compared to single-species controls ($P > 0.05$). For this reason, further analysis compared expression in mature 24-h single- and dual-

species biofilms only. Heat map analysis of normalized \log_2 fold change in gene expression between single- and dual-species 24-h biofilms with *L. crispatus* revealed upregulation of many genes involved in amino acid biosynthesis and breakdown in dual-species biofilms (*ARG8*, *ILV1*, and *HIS5*) (Fig. 7). Interestingly, the *PRY1* gene, which codes for a secreted protein associated with virulence in the presence of lactate in *C. albicans*, was found to be downregulated in dual-species biofilms (\log_2 fold change = -25.35). A list of some of the key genes and their functions is presented in Table 1. Gene ontology (GO) term analysis was utilized to determine the functionality of differentially expressed genes in single- and dual-species 24-h biofilms.

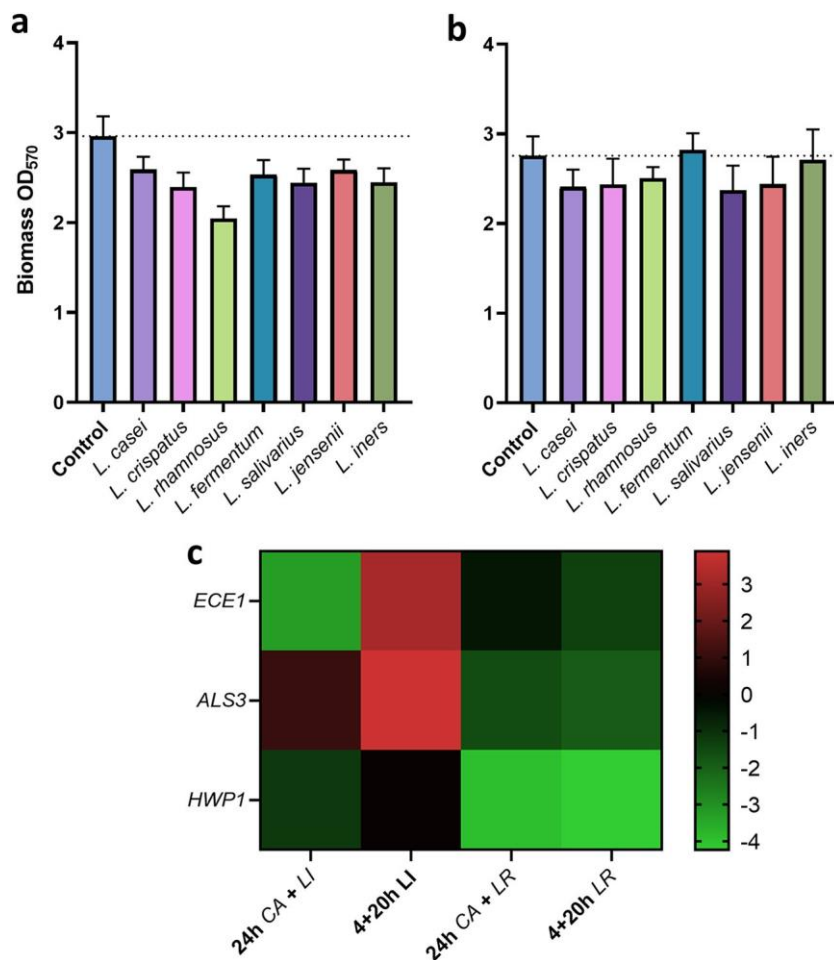


FIG 6 *Lactobacillus* species display antagonism with *C. albicans* *in vitro*. To observe inhibitory effects of *Lactobacillus* against *C. albicans* biofilm formation, *C. albicans* and a panel of *Lactobacillus* species were cocultured in THB/RPMI (1:1) medium in 5% CO₂ for 24 h (a), or *C. albicans* was grown for 4 h prior to addition of *Lactobacillus* species for 20 h (b). *C. albicans* biofilm-associated gene expression was measured in the presence of *L. rhamnosus*, which is associated with health, and *L. iners*, which is hypothesized to indicate dysbiosis. The mean log fold change relative to single species *C. albicans* biofilms is shown (c). Data are means and SD.

Genes expressed in *L. crispatus* dual-species biofilms were mainly responsible for amino acid biosynthesis and some metabolic and transaminase activity (Fig. 8a and b). Despite upregulation of these amino acid biosynthesis and metabolism pathways in *C. albicans*, expression of *BAT21* and *ILV1* in dual-species biofilms suggests that *C. albicans* was in a state of amino acid starvation (Fig. 8c).

Given the antagonism observed between the two organisms, we next aimed to investigate the *in vitro* probiotic potential of *L. crispatus* against *C. albicans* infection in a complex biofilm model (Fig. 9). After 2 consecutive days of probiotic treatment, a slight reduction in total and live *C. albicans* composition within the biofilm was observed; however, this was not significant ($P = 0.55$ and $P =$

0.16, respectively) (Fig. 9a and b). Following a 4-day treatment regimen with *L. crispatus*, total *C. albicans* composition decreased ($P < 0.05$), with reduced levels of live fungal DNA. When the total fold reduction in *C. albicans* from untreated biofilms was assessed, the greatest probiotic effect was observed at 48 h posttreatment (Fig. 9c).

DISCUSSION

Unlike systemic and oral candidiasis, RVVC affects immunocompetent women with an incidence rate of up to 8% in women of child-bearing age; hence, it is the most

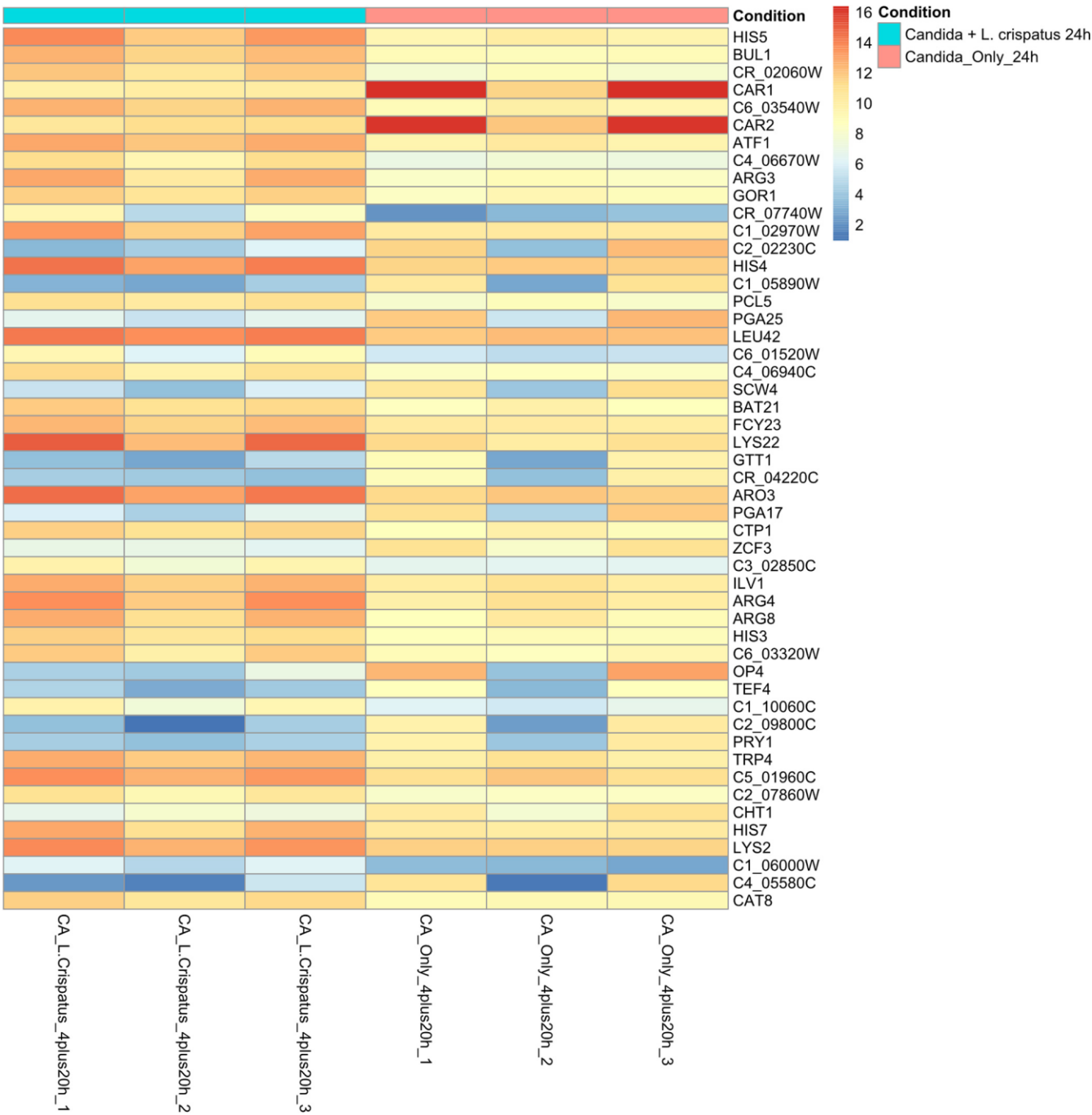


FIG 7 Differential expression analysis of *C. albicans* single-species and *C. albicans*–*L. crispatus* dual-species biofilms. Heat map displaying the top 50 significantly differentially expressed genes in *C. albicans* between single- and dual-species 24-h biofilms ($P < 0.05$).

prevalent human infection caused by the pathogenic yeast, with approximately 140 million cases annually (36). Despite this, the disease remains largely understudied in the field of women's health. A better understanding of RVVC at a molecular level may be crucial in unearthing potential targets for much-desired therapeutics. This study aimed to assess a panel of 100 clinical

isolates to investigate fungal influence and changes in bacterial communities and to use an RNA sequencing approach to analyze potential interkingdom interactions contributing to pathology.

Clinical and microbiological assessment of patient samples. The estimated vaginal commensal carriage rate of *Candida* is 33% (9). We observed the presence of

TABLE 1 Upregulated genes in 24-h dual-species biofilm associated with amino acid biosynthesis and/or breakdown

Gene name	Function	Log ₂ fold change
<i>arg3</i>	Arginine and citrulline biosynthesis	3.72897
<i>his5</i>	Histidine biosynthesis	3.47441
		5
<i>lys22</i>	Lysine biosynthesis	3.46901
		3
<i>bul1</i>	Protein ubiquitination	3.25467
		1
<i>arg4</i>	Arginine biosynthesis/metabolism	2.81015
		8
<i>atf1</i>	<i>N</i> -Acetyltransferase activity	2.70428
		1
<i>arg8</i>	Arginine biosynthesis/metabolism	2.69588
		5
<i>aro3</i>	Phenylalanine, tyrosine, and tryptophan biosynthesis	2.41157
		3
<i>his4</i>	Histidine, purine, and pyrimidine biosynthesis	2.35265
		4
<i>his3</i>	Histidine, purine, and pyrimidine biosynthesis	2.34044
		6
<i>bat21</i>	Leucine, valine, and isoleucine biosynthesis/breakdown	2.28467
		7
<i>ilv1</i>	Leucine, valine, and isoleucine biosynthesis, threonine breakdown	2.04984
		5
<i>leu42</i>	Leucine, valine, and isoleucine biosynthesis	1.94549
		6

Candida both by culture and by qPCR and found that 15% and 60% of samples from healthy women and those with RVVC had culturable *Candida*, respectively, and all but 2 samples were positive by qPCR. As expected, levels of the inflammatory biomarker IL-8 and *Candida* DNA were found to be significantly higher in patients with RVVC, confirmatory of clinical diagnosis. It is important to note that these clinical samples are from women regularly attending sexual health clinics, and those who have suffered from RVVC for longer may be more likely to be receiving antifungal treatment. This should be considered when interpreting *Candida* CFE/ml data in this study, as untreated RVVC may present differently.

Taxonomic classification of health and RVVC. Unlike the vaginal microbiome during bacterial vaginosis (BV), the microbial communities present during VVC have been shown to be similar to those present in healthy women at the phylum and genus levels (37, 38). Our study confirms these findings, as we report a *Lactobacillus*-dominated population with vaginal anaerobes, including *Gardnerella*, *Prevotella*, and *Atopobium*, with no significant differences in diversity or composition between the two cohorts at the genus level. This suggests that the functional capacity of the bacterial species found in healthy women and those with RVVC may play a more

crucial role in pathology. We observed a reduction in specific *Lactobacillus* species, including those associated with maintaining health due to their ability to produce L-lactic acid and H₂O₂, such as *L. crispatus* and *L. jensenii*. This reduction coupled with an increase of *L. iners* has been shown previously and is thought to be indicative of vaginal dysbiosis (37). We report that this loss of health-associated *Lactobacillus* species and increase of *L. iners* is also seen specifically in patients with positive *Candida* cultures and those who have suffered from RVVC for ≥ 6 months.

We show an *L. iners*-dominated microbiome in women using contraceptive devices or no contraception, which suggests that hormonal contraceptives may be less dysbiotic to the vaginal flora or have the potential to maintain a more health-like microbiome than contraceptive devices. Additionally, recent antifungal use revealed a microbial profile similar to those of healthy patients, suggesting that, although somewhat effective, the current antifungal treatments for RVVC do not alter the microbiome to a sufficient level to reintroduce a health-like state. This is evident with the microbiome reverting to favor a disease-like environment after less than 1 month. This is similar to results found by other authors, who reported a transition from disease to an intermediate state following fluconazole treatment (16). Although not associated with vaginal dysbiosis, the genus *Shuttleworthia* was positively correlated with *Candida* load and increased time between treatments and is often observed in high levels in vaginal microbiome studies (39). Regardless of disease status, *Lactobacillus* was negatively correlated with the presence of *Candida* as well as increased numbers of episodes and time between treatments,

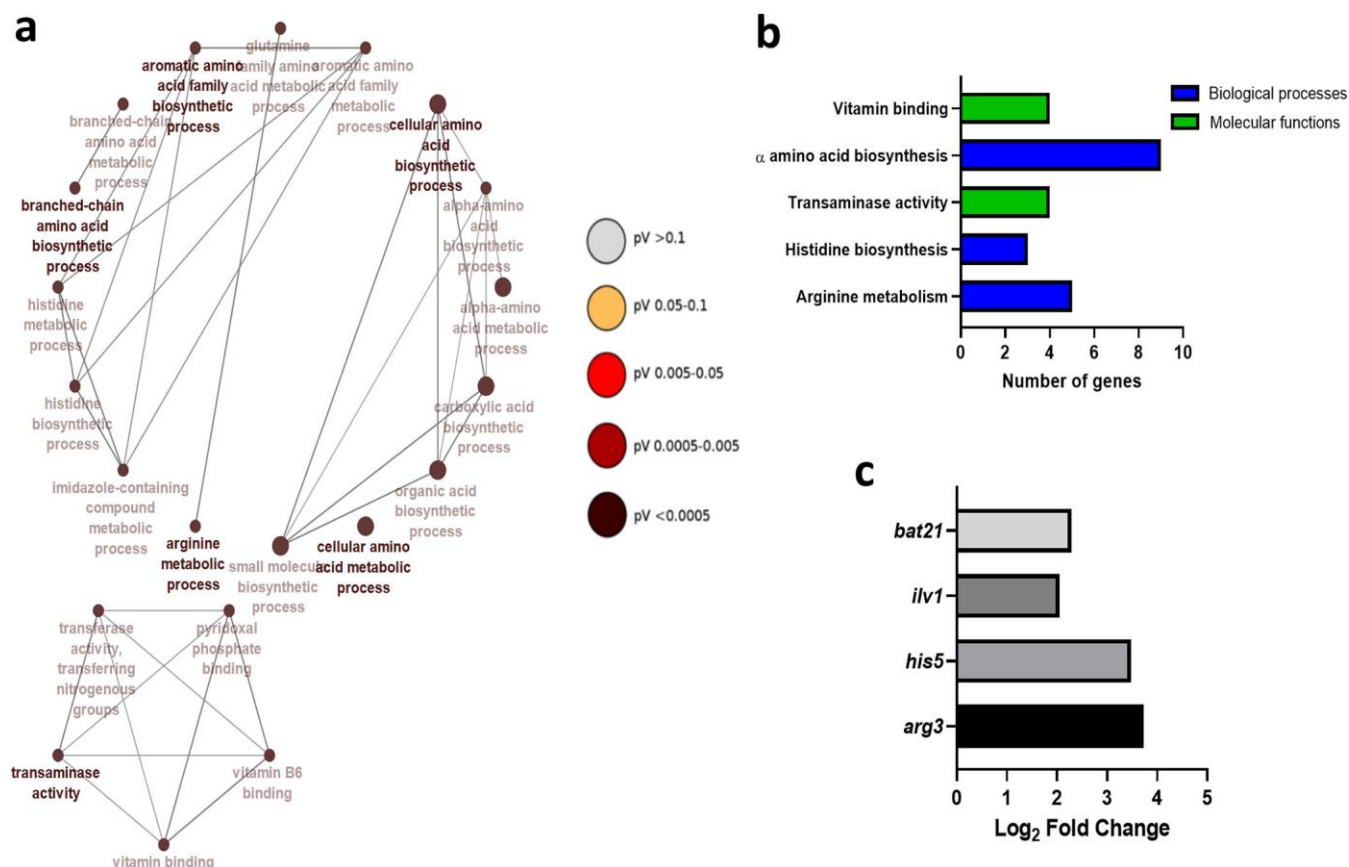


FIG 8 Gene networks of gene ontology (GO) terms and upregulated genes in dual-species biofilms. Constructed gene networks of GO terms in 24-h biofilms with *L. crispatus* (a). Important biological, cellular, and molecular functions in dual-species biofilms with *L. crispatus* (b). Log₂ fold change of key gene expression in *C. albicans* from single- to dual-species biofilms with *L. crispatus* (c). Nodes are colored by significance. All GO terms have an adjusted *P* value of ≥ 0.05 . Networks were created using ClueGO.

indicating the potential protection from *Candida* overgrowth provided by vaginal lacto-bacilli. These findings may be important for future studies investigating RVVC. It is hypothesized that changes within *Candida* allow it to switch from asymptomatic commensal to pathogenic yeast. It may now be more important to study RVVC with a specific focus on the microbes present during RVVC, specifically *Lactobacillus*, and interkingdom interactions which may influence this pathogenic switch in *Candida*.

Influence of *Candida* biofilm formation in RVVC. Consistent with epidemiological reports, we observed *C. albicans* to be the most common isolate identified, followed by *C. glabrata*. Though *C. albicans* has been shown to form biofilms on vaginal mucosa and *Candida* clinical isolates have been shown to form biofilms *in vitro*, the presence of biofilms during RVVC infection is still disputed (37). Despite evidence to the contrary, our study has shown heterogeneous biofilm formation in *C. albicans* RVVC isolates and shows clear visualization of *C. albicans* hyphae and

aggregates in lavage fluid from a patient with RVVC. Moreover, transcripts from genes associated with biofilm formation were readily detected from vaginal lavage fluid. Therefore, *Candida* biofilm formation within the vagina should not be discounted as a potential cause of failed clinical treatment and subsequent recurrence of disease. We also show the capacity of various *Lactobacillus* species to inhibit *C. albicans* biofilm formation when cocultured. *L. rhamnosus* has been studied extensively for its potential as a probiotic and has been shown to prevent adhesion of *C. albicans* to mucosal surfaces (39). Here, we show the ability of *L. rhamnosus* to down-regulate *C. albicans* biofilm-related gene expression. In contrast, *L. iners* results in upregulation of both *ALS3* and *ECE1*. This is consistent with previous findings suggesting that *L. iners* may be indicative of a dysbiotic vaginal environment and should not be considered as a probiotic intervention for *C. albicans* infections (40).

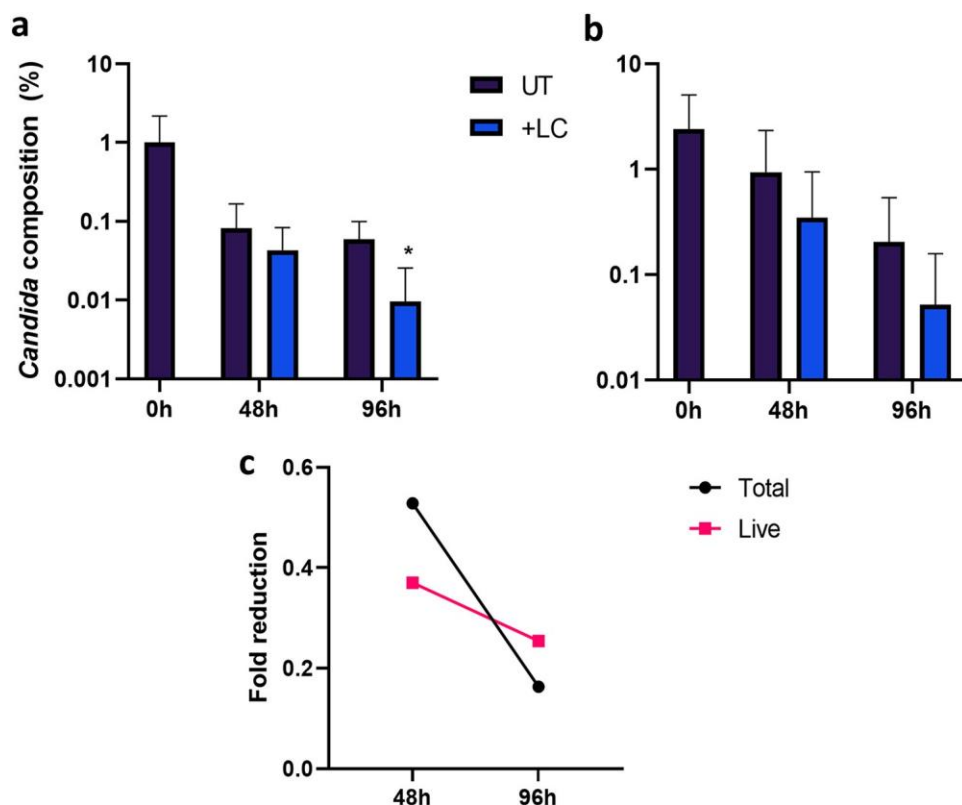


FIG 9 Twice-daily addition of *L. crispatus* reduces *C. albicans* load within a complex biofilm model *in vitro*. The potential probiotic properties of *L. crispatus* against *C. albicans* were assessed in a 11-species biofilm model treated twice daily with *L. crispatus*. Live/dead qPCR allowed quantification of percentage composition of total (a) and live (b) *C. albicans* DNA within the biofilm. Average fold change in the *C. albicans* percentage composition from the untreated 11-species biofilm is also shown (c). Data are means and SD. Statistical analysis was performed using paired *t* tests comparing raw CFE values (*, *P* < 0.05).

Antagonism of *Lactobacillus* and *C. albicans*. In our transcriptomic analyses we aimed to examine mechanisms of reported antagonism between *C. albicans* and *L. crispatus*. It has previously been shown in *Lactobacillus* clinical isolates that secreted metabolites can remain at low levels from 72 or 120 h of incubation, which may account for the delay in interaction (41). The *in vivo* vaginal pH is thought to be ~4.5; however, in this dual-species model, the pH was sustained between 6 and 7. It is possible that in the *in vivo* setting, the lower environmental pH could affect *C. albicans* gene expression. Nonetheless, the stability of the neutral pH throughout this experiment dictates that any transcriptomic changes observed are not due to acidification of the medium and are a result of interactions between *C. albicans* and *L. crispatus*.

Transaminase activity is primarily associated with α -amino acid breakdown and bio-synthesis. It has been shown in a model of *Saccharomyces cerevisiae*, *L. lactis*, and *L. plantarum* that in nitrogen-rich environments, yeasts secrete an array of metabolites,

primarily amino acids, thereby facilitating the growth of lactobacilli (42, 43). The upregulation of various amino acid biosynthesis processes in our study may be attributed to this nutrient cross-feeding. As opposed to a convenient coincidence, this may be a deliberate process by which *L. crispatus* is able to drive synthesis of amino acids in *C. albicans*, as well as suppressing arginine breakdown, to facilitate its own metabolism and growth. The upregulation of the amino acid starvation indicators *bat21* and *ilv1* in dual-species biofilms shows that despite the upregulation of various amino acid biosynthesis processes, *C. albicans* is unable to utilize them. This may be a contributing factor in the process by which *L. crispatus* is able to outcompete *Candida* during VVC and potentially re-establish a healthy microbiome. Furthermore, the *pry1* gene, encoding a secreted glycoprotein associated with virulence and sterol binding in the

presence of lactate, was found to be downregulated in dual-species biofilms (44). Thus, *L. crispatus* may possess a mechanism by which it can suppress lactate-associated virulence in *C. albicans*. Species-specific differential transcriptomic profiles in vaginal candidiasis have been demonstrated, and therefore, interactions between *Lactobacillus* and other prevalent *Candida* species should be considered (44). These data collectively show a potentially probiotic effect of *L. crispatus* against *C. albicans* in nonacidic environments. This antagonism is governed by the overproduction of amino acids from *C. albicans*, which may facilitate restoration of a healthy microbiome through *Lactobacillus* proliferation.

Finally, we investigated the potential probiotic effect of *L. crispatus* against *C. albicans* within a complex biofilm model. Although studies have assessed the inhibitory effect of

L. crispatus against *C. albicans* monospecies biofilms (33, 45, 46), to our knowledge, this is the first report of *L. crispatus* reducing *C. albicans* composition within a polymicrobial biofilm model *in vitro*. Our data support other studies confirming the probiotic potential of *L. crispatus* against *C. albicans* biofilm infections.

This study assessed the vaginal microbiome only of women in the United Kingdom; results may differ based on geographical prevalence of different *Candida* and *Lactobacillus* species. Additionally, a larger sample set may allow for statistically significant differences in the microbiome of women with VVC and healthy controls. It may be useful for future studies to observe transcriptional changes of *C. albicans* with other health-associated vaginal *Lactobacillus* species, such as *L. jensenii* and *L. gasseri*.

In conclusion, *Candida* burden is increased in women with RVVC compared to asymptomatic controls and continues to increase following antifungal intervention due to resistant communities. The reduction of protective H₂O₂-producing *Lactobacillus* species during RVVC is exacerbated in women who have RVVC for longer than 6 months, those who have culturable *Candida*, those who use no contraception or a contraceptive device, and those who are treated less frequently. Additionally, demonstration of biofilm-forming capabilities and imaging of *C. albicans* aggregates in patient lavage fluid, as well as the expression of *C. albicans* biofilm-related genes, suggest that there is a high probability of *Candida* biofilm formation in the vagina during RVVC. The subsequent increased antifungal tolerance of these biofilms may allow *Candida* to persist within the vagina and cause recurrence of VVC. Our study suggests that it may be important to view RVVC as a result of fluctuation in antagonistic interkingdom interactions between *Candida* and *Lactobacillus*,

potentially as a result of *Candida* biofilm formation. Additionally, we show for the first time the potential of *L. crispatus* to re-establish a healthy vaginal environment through altered gene expression in *C. albicans* and the ability to reduce fungal composition in a complex biofilm model. Lactobacilli are key protective organisms in the healthy vaginal microbiome, and their antagonistic effect on *Candida* is of clinical significance. As demonstrated within our clinical study, there was a decrease in levels of protective *Lactobacillus* species in RVVC patients. The decrease of levels of these species also inversely correlated with an increase in fungal load. Further understanding of this antagonism offers potential avenues for improving women's health through probiotic/prebiotic regimens. This interaction could prove impactful in the development of novel mechanisms for antifungal treatments, and therefore, further studies of this interaction are required.

MATERIALS AND METHODS

Patient recruitment and collection of clinical samples. One hundred women aged 18 and over attending Glasgow Sandyford Sexual Health Clinic were enrolled in the study. Patient recruitment and obtaining of written informed consent were carried out at the clinic from prospective participants after they had read the patient information sheet. If the patient chose to participate, a questionnaire was completed and given to the clinician in charge of the study (R.M.). Patients with current, confirmed symptomatic RVVC ($n = 40$) were recruited as well as asymptomatic women attending the clinic for contraceptive intrauterine device (IUD) implantation, acting as a healthy cohort ($n = 60$). This study was granted ethical approval by the Sheffield Research Ethics Committee (16/YH/0310). Patients were excluded from the study if they had prior or active bacterial vaginosis, were pregnant, immunosuppressed, menstruating, or menopausal, or had taken antibiotics/antifungals within 7 days prior to sampling. From each patient, one high vaginal swab (HVS) and one cervicovaginal lavage fluid sample (CVL) were collected. A graphical representation of sample collection and processing can be found in Fig. S1.

Detection of inflammatory biomarkers in CVL fluid. CVL fluid supernatants were recovered by centrifugation. Levels of IL-8 were measured by enzyme-linked immunosorbent assay (ELISA) (Invitrogen, Paisley, UK), following the manufacturer's instructions. CVL fluid was diluted 1:2, and absorbances were measured using a spectrophotometer at 450 nm and 570 nm.

Quantification of microbial load by quantitative PCR. HVS samples were used to extract DNA for quantitative PCR (qPCR) analysis of *Candida*/bacterial burden. DNA was extracted using the QIAamp DNA minikit, per the manufacturer's instructions (Qiagen, Crawley, UK), and qPCR was used to quantify fungal and bacterial load in each sample. Primers specific to the conserved *Candida* internal transcribed spacer (ITS) rRNA gene (47) were used to determine *Candida* load. For bacterial load, primers and probe specific to the 16S rRNA gene were used (48, 49). Primer sequences can be found at <https://www.glasgowbiofilms.co.uk/>. The total qPCR volume was 20 μ l, with 1 μ l of extracted DNA, 500 μ M forward/reverse primers, and UV-treated RNase-free H₂O. For 16S, 250 μ M probe and 2 TaqMan universal PCR master mix (Thermo Scientific, Loughborough, UK) was used; 2 Fast SYBR green PCR master mix (Thermo Scientific, Loughborough, UK) was used for ITS primers. qPCR was carried out using a Step-One Plus real-time PCR machine (Life Technologies, Paisley, UK) with the following thermal conditions: an activation step of 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 10 s to denature and annealing at 60°C for 30 s. Standard curves constructed from serially diluted DNA of *C. albicans* SC5314 and *Escherichia coli* K-12 were used to extrapolate *Candida* and bacterial colony-forming equivalents (CFE) per milliliter, respectively, as described previously (50).

Preparation of 16S amplicon libraries for Illumina sequencing. To observe bacterial populations in the samples, 16S rRNA sequencing was performed. Briefly, previously extracted HVS DNA was used to sequence the 16S rRNA V4 region using the Illumina MiSeq sequencing platform (Edinburgh Genomics) using 2 x 250-bp paired-end reads. Amplification of the V4 region was achieved using fusion Golay adaptors barcoded on the reverse strand as described previously (51) using the universal primer 515F (59-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-39) and reverse primer 806R (59-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-39). Quality control (QC) of reads was performed within the R package DADA2 (v1.14.1). Reads were filtered and trimmed using standard error rates before reads were denoised and merged and chimeric sequences removed. Amplicon sequence variants (ASV) were annotated by the Silva 132 16S rRNA database with an exact match to the ASV. ASV and taxonomic assignments were utilized for downstream data analysis.

Identification and biofilm screening of *Candida* species from cervicovaginal lavage fluid. Lavage fluid samples were screened for presence and identification of *Candida* species using Colorex *Candida* chromogenic agar (E&O Laboratories Ltd., Bonnybridge, UK) and matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry. For culture identification, 20 μ l of CVL samples was spread across the surface of a chromogenic agar plate before 48 h of incubation at 30°C. The color of the colonies cultured was used to determine *Candida* species, colony numbers were used to calculate the number of CFU per milliliter. Clinical isolates were then stored on beads in glycerol in Microbank vials (Pro-Lab Diagnostics, Cheshire, UK) at 280°C. Each isolate was then subsequently identified by MALDI-TOF analysis using a Bruker Microflex system, comparing recorded spectra to the Bruker database to confirm identity to the species level.

Candida clinical isolates were assessed for biofilm-forming capabilities by crystal violet assay. *Candida* isolates ($n = 33$) were cultured on Sabouraud's (SAB) agar for 48 h at 30°C. For biofilm formation, overnight cultures were grown in yeast extract peptone dextrose (YPD) at 30°C. Cultures were washed twice with phosphate-buffered saline (PBS) and standardized in RPMI 1640 medium to a final cell density of $\approx 10^6$ CFU/ml. Eight biofilms of each isolate were grown in 96-well, flat-bottomed polystyrene microtiter plates for 24 h at 37°C before biomass measured by the crystal violet assay (52). For visualization of *Candida* aggregates, 30 μ l of CVL was stained with calcofluor white (CFW) to a final concentration of 0.06 μ g/ml for 1 h at 37°C, before being imaged using the EVOS live cell imaging system (Thermo Scientific, Loughborough, UK).

Antagonism of *C. albicans* and *Lactobacillus* in coculture. The ability of the following 7 *Lactobacillus* strains to inhibit *C. albicans* SC5314 biofilm formation was assessed: *L. casei* ATCC 393, *L. fermentum* ATCC 14931, *L. crispatus* ATCC 33820, *L. iners* DSMZ 13335, *L. salivarius* ATCC 11741, *L. jensenii* ATCC 25258 and *L. rhamnosus* ATCC 7469. Overnight cultures of *C. albicans* and *Lactobacillus* were grown in YPD and de Man, Rogosa, and Sharpe (MRS; Merck UK) medium, respectively, under appropriate culture conditions. For these experiments, we used an optimized medium of Todd-Hewitt broth (THB; Merck UK) supplemented with 10 μ M menadione and 10 μ g/ml hemin (Thermo Fisher) and mixed 1:1 with RPMI (referred to here as 1:1 broth), as described previously for coculture experiments (53). For biofilm formation, overnight cultures were standardized to 1×10^6 CFU/ml for *C. albicans* and 1×10^7 CFU/ml for *Lactobacillus* species in 1:1 medium. Eight biofilms of each *C. albicans*-*Lactobacillus* pair were incubated in 5% CO₂ for 24 h. In addition, *C. albicans* biofilms were grown for 4 h prior to *Lactobacillus* being added for 20 h, with biomass quantified using the crystal violet assay.

C. albicans biofilm-related gene expression analysis. To assess *C. albicans* biofilm-related gene expression, RNA was extracted from dual-species biofilms using the PureLink RNA minikit (Thermo Scientific, Loughborough, UK), following manufacturer's instructions. In brief, 2 μ g of RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit (Thermo Scientific, Loughborough, UK) and 1 μ l was used in a 20- μ l qPCR with 10 μ l 2x Fast SYBR green PCR master mix, 1 μ l forward/reverse primer, and UV-treated H₂O. Primer sequences can be found at <https://www.glasgowbiofilms.co.uk/> (54–56). Gene expression was analyzed in duplicate on three separate occasions; no-reverse-transcription (NRT) controls and no-template controls (NTC) were included throughout. Gene expression was normalized to the *ACT1* housekeeping gene and calculated using the DDC method (57).

In vitro transcriptomic analysis of *C. albicans* interactions with *L. crispatus*. *C. albicans* and *L. crispatus* type strains SC5314 and ATCC 33820, respectively, were used for transcriptional analysis in this component of the study. Biofilms were formed as described above. Cultures were washed twice with PBS and standardized in 1:1 broth to 1×10^6 CFU/ml for *C. albicans* and 1×10^7 CFU/ml for *L. crispatus*. Initially, *C. albicans* biofilms were grown in T-75 cell culture flasks (Corning, USA) for 4 h in 5% CO₂. Following incubation, medium was removed, and biofilms washed before *L. crispatus* was added for an additional 2, 4, or 20 h. At each time point, the medium was removed and biofilms were washed with PBS before being scraped into 1 ml of RNeasy lysis buffer (Thermo Scientific, Loughborough, UK). Spent medium from biofilms was retained, and pH was monitored throughout the experiment. RNA was extracted from microbial biofilms using the RiboPure RNA purification kit for yeast (Thermo Scientific, Loughborough, UK), following manufacturer's instructions. Integrity of RNA was assessed using a Bioanalyzer system, and genome-wide *Candida* transcripts were sequenced using the Illumina NOVASeq6000 sequencing platform (Edinburgh Genomics). FastQC was used to assign quality scores to the produced reads, and Illumina adaptors and poor-quality reads were trimmed using Trimmomatic (V0.38). Hisat2 (v2.1.0) was then used to align the resulting reads to a reference *C. albicans* genome (<http://www.candidagenome.org/>) before the number of sequences that were aligned to each gene was counted using HTSeq-count (v0.11.0). The counted genes were subsequently imported into RStudio, and the DESeq2 package was used to analyze the differentially expressed genes. A summarized illustration of the experimental and bioinformatics pipe-lines can be found in Fig. S1b and c.

Investigating the probiotic potential of *L. crispatus* in a complex biofilm model. Complex 11-species biofilms were formed as described previously by our group with slight modifications (58). Overnight broths of each organism were standardized in 1:1 medium prior to addition to the biofilm on 13-mm discs. For probiotic treatment, each biofilm was treated twice daily (12 h intervals) with 5×10^7 CFU/ml of *L. crispatus* for 5 min before treatment was removed, biofilms washed 3 times in PBS and fresh media replaced. On day 3, after 4 probiotic treatments, 48-h biofilms were analyzed for compositional analysis. On day 5, following 8 probiotic treatments, 96-h biofilms were removed for compositional analysis. At each time point, biofilms were washed 3 times and sonicated in 1 ml of PBS at 35 kHz for 10 min to remove biomass. Sonicates were split in two, with one sample having 5 ml of 10 mM propidium monoazide (PMA) added to it for the quantification of live *C. albicans* DNA. PMA is a DNA-intercalating dye used to bind DNA from dead cells or those with a compromised membrane following exposure to a halogen light (59, 60). The other sample, lacking PMA, allowed quantification of total *C. albicans* DNA per biofilm. All samples were incubated in the dark for 10 min, then placed on ice, and exposed to a 650-W halogen light for 5 min. DNA was then extracted using the QIAamp DNA minikit, per the manufacturer's instructions (Qiagen, Crawley, UK). To a 20- μ l qPCR mixture, 1 μ l of biofilm DNA was added; the mixture contained 10 μ l Fast SYBR green master mix, 1 μ l of 10 mM *C. albicans* forward and reverse primers, and UV-treated nuclease-free water. Primer sequences can be found at <https://www.glasgowbiofilms.co.uk/>. The following thermal profile was used: 95°C for 2 min and 40 cycles of 95°C for 3 s followed by 55°C for 30 s. Samples were assessed in duplicate from 2 separate experiments. Fungal CFE/ml were then calculated as described above.

Statistical analysis. Microbiome figures were created using MicrobiomeAnalyst (61). Bacterial diversity and abundance plots were made using alpha diversity and stacked-bar charts, respectively. Random forest plots were used to predict important species present under each condition. Transcriptome pipe-line figures were constructed using BioRender and differential gene expression plots using the DESeq2 package in RStudio. Gene ontology networks were constructed using ClueGO software, available through Cytoscape (62). All other figures and analyses were performed in GraphPad Prism (version 8; GraphPad, La Jolla, CA, USA).

Data availability. Microbiome data are deposited under the accession number [PRJNA719953](https://www.ncbi.nlm.nih.gov/sra/PRJNA719953) in the SRA database.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.2 MB.

FIG S2, TIF file, 0.1 MB.

FIG S3, TIF file, 0.1 MB.

FIG S4, TIF file, 0.2 MB.

FIG S5, TIF file, 0.2 MB.

FIG S6, TIF file, 0.1 MB.

FIG S7, TIF file, 0.1 MB.

FIG S8, TIF file, 0.1 MB.

TABLE S1, DOCX file, 0.1 MB.

TABLE S2, DOCX file, 0.02 MB.

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E.M., C.D., L.S., R.K., and S.W. participated in study design and experimental procedures. E.M., C.D., L.S., and R.K. were responsible for preparation of the manuscript. L.S., R.M., and R.T. were responsible for ethics approval, clinical sample collection, and collection of patient metadata. C.W., C.J.N., R.R., and K.G. participated in study design and contributed to the manuscript. G.R. conceived the study, participated in study design, and was responsible for producing the final manuscript. All authors read and approved the final manuscript.

We declare no conflict of interest.

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